The Cation-dependent Mannose 6-phosphate Receptor is S-palmitoylated and Ubiquitinated for its Trafficking in Endosomes

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Bruno G.H. Filippi

aus

Frankreich

Promotionskomitee

Prof. Dr. Thierry Hennet (Vorsitz)

Prof. Dr. Jack Rohrer (Leitung der Dissertation)

Prof. Dr. Eric G. Berger

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List of abbreviations

ABE	Acy-biotin exchange
bCD-MPR	Bovine cation-dependent mannose 6-phosphate receptor
Biotin-HPDP	*N*-(6-(Biotinamido)hexyl)-3'-(2'-pyridyldithio)-propionamide
BSA	Bovine serum albumin
Cav1	Caveolin 1
CD-MPR	Cation-dependent mannose 6-phosphate receptor
cDNA	Complementary deoxyribonucleic acid
CI-MPR	Cation-independent mannose 6-phosphate receptor
ddH_2O	Doubled distilled water
DAPI	4',6-diamidino-2-phenylindole
[DE]XXXL[LI] protein motif	[Asp/Glu]-X-X-X-Leu-[Leu/Ile] protein motif where X is any amino acid
DHHC protein motif	Asp-His-His-Cys protein motif
DXXLL protein motif	Asp-X-X-Leu-Leu protein motif where X is any amino acid
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DRM	Detergent resistant membrane
DSM	Detergent sensitive membrane
DTT	Dithiothreitol
DUB	Deubiquiting enzyme
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERGIC	Endoplasmic reticulum Golgi intermediate compartment
ESCRT	Endosomal sorting complex required for transport
FCS	Fetal calf serum
FTase	Farnesyltransferase
FXNPXY protein motif	Phe-X-Asn-Pro-X-Tyr protein motif where X is any amino acid
FYVE domain	Fab-1, YGL023, Vps27, and EEA1 domain
GA	Golgi apparatus
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine di-phosphate
GEF	Guanine nucleotide exchange factor
GGTase	Geranylgeranyltransferase
GPBS	Phosphate buffered saline supplemented with 5 mM glycine
GPCR	G-protein-coupled signaling receptor
GPI	Glycosylphosphatidylinositol

GTP	Guanosine tri-phosphate
HRP	Horseradish peroxidase
IgG	Immuno-globulin G
ILV	Intra-luminal vesicle
KDEL protein motif	Lys-Asp-Glu-Leu protein motif where X is any amino acid
KKXX protein motif	Lys-Lys-X-X protein motif where X is any amino acid
KXKXX protein motif	Lys-X-Lys-X-X protein motif where X is any amino acid
LBPA	Lysobiophosphatidic acid
LE	Late endosome
MPBS	Phosphate buffered saline supplemented with three percent low-fat powder milk (weight pro volume)
MPR $C^{30}C^{34}$-A	Palmitoylation-deficient mutant form of the bovine cation-dependent mannose 6-phosphate receptor
MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A	Internalization-deficient mutant form of the bovine cation-dependent mannose 6-phosphate receptor
MPR $K^{8}K^{37}$-R	Ubiquitination-deficient mutant form of the bovine cation-dependent mannose 6-phosphate receptor
mRNA	messenger ribonucleic acid
MVB	Multi-vesicular body
NEM	N-ethylmaleimide
NMT	N-myristoyltransferase
NSF	N-ethylmaleimide-sensitive fusion protein
PAT	Protein acyl transferase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIC	Protein inhibitor cocktail
PI3P	Phosphatidylinositol 3-phosphate
PFA	Paraformaldehyde
PM	Plasma membrane
PMSF	Phenylmethanesulphonylfluoride
PVDF	Polyvinylidene fluoride
PX domain	Phox domain
Ras-like protein	Proteins classified into the rat sarcoma protein super-family
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Real time polymerase chain reaction
SB	Sample buffer
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SigmaPIC	Protein inhibitor cocktail obtained from Sigma-Aldrich
siRNA	Small interfering ribonucleic acid
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
SNX	Sorting nexin
sulfo-NHS-biotin	Sulfosuccinimidobiotin
TBS	Tris-buffered saline
TBST	Tris buffered saline supplemented with 0.05% of TWEEN20
TCA	Trichloroacetic acid
TfR	Transferrin receptor
TGN	Trans-Golgi network
TMD	Trans-membrane domain
TNE	Tris-buffered saline supplemented with ethylenediaminetetraacetic acid
UIM	Ubiquitin interacting motif
v/v	Volume pro volume
w/v	Weight pro volume
Wt or WT	Wild type
YXXθ protein motif	Tyr-X-X-θ protein motif where X is any amino acid and θ a hydrophobic amino acid

General foreword on the intracellular trafficking of biomolecules

According to Erwin Schrödinger, a feature of the living systems, and the most important to our eyes, is the ability to maintain their own entropy low. And what is a living system if it is not a system of ordinary elements organized in such a way that it acquires control over its own entropy?

The smallest system complying with such a simple but demanding principle is the cell, which is the structural and functional unit of all living organisms. “Living organisms”, this name underscores one of the most critical features allowing living systems to simple exist: “organization”.

It is through a complex and tightly regulated organization of its components that the cell maintains its homeostasis, controls its metabolism, monitors its growth or adapts to the environment.

Cells are lipid bilayer closed systems, a feature which allows the physical isolation of the regulated intra-plasma membrane environment (cytoplasm) from the unregulated extra-plasma membrane environment (extracellular milieu). Within the cytoplasm, the eukaryotic cell disposes of specialized compartments which fulfill discrete functions crucial to its survival. To fulfill their functions, those compartments require a specific set of biomolecules which bear structural, enzymatic or distinctiveness roles. Consequently, understanding how molecules are able to efficiently traffic to their proper intracellular localization is crucial for the field of cellular biology.

Summary

The lysosomes form a crucial cellular compartment which degrades cellular wastes, and recycles the digested parts for the biosynthesis of new components. The biological importance of the lysosomes for the cell as well as for the entire organism is best illustrated by the fact that the disturbance of lysosomal functions leads to severe metabolic diseases such as mucolipidosis, Gaucher's disease or Niemann-Pick's disease. The cation-dependent mannose 6-phosphate receptor sustains lysosomal function by redirecting newly synthesized lysosomal enzymes from the secretion pathway to the lysosomes. To fulfill this task, the cation-dependent mannose 6-phosphate receptor captures lysosomal enzymes within the trans-Golgi network, transports them to endosomes where the enzymes are released, and recycles back to the trans-Golgi network for a new round of sorting. The proper sorting of the receptor within endosomes is crucial to avoid the aberrant transport of the receptor to lysosomes where it would be degraded. Interestingly, the post-translational modification S-palmitoylation has previously been shown to be essential for the proper sorting of the receptor in endosomes. In addition, a protein acyl transferase activity for the cation-dependent mannose 6-phosphate receptor has been found to exist at the plasma membrane and in endosomes.

In this work, we studied two post-translational modifications (i.e. S-palmitoylation and ubiquitination) and their role for the correct sorting of the cation-dependent mannose 6-phosphate receptor within endosomes. It is shown that the receptor ubiquitinated on its cytosolic tail, and we give evidence that this modification is part of a quality control mechanism occurring at the plasma membrane and endosomes. In addition, it was demonstrated in this work that S-palmitoylation allows the association of the receptor with cholesterol rich membrane sub-domains which most probably are localized in early endosomes. With the knowledge that cholesterol rich

membrane sub-domains are important for the retrieval of the cation-dependent mannose 6-phosphate receptor from the endosomes to the trans-Golgi network, S-palmitoylation appears to be a molecular switch which allows the association of the receptor with cholesterol rich membrane sub-domains in endosomes to promote its retrieval to the trans-Golgi network. In order to identify the enzyme catalyzing the S-palmitoylation of the cation-dependent mannose 6-phosphate receptor, a new family of protein acyl-transferases (the ZDHHC protein family) was analyzed. Consequently, a new phylogeny of ZDHHC protein family was established, the intracellular localization of individual ZDHHC proteins was determined, and their enzymatic activity for the cation-dependent mannose 6-phosphate receptor was analyzed.

As a result of this PhD work, a new model for the trafficking of the cation-dependent mannose 6-phosphate receptor within endosomes is proposed, and a restricted number of ZDHHC proteins are identified as putative protein acyl-transferases for the cation-dependent mannose 6-phosphate receptor. In addition, the phylogenetic analysis of the ZDHHC protein family revealed groups of ZDHHC proteins with a close evolutionary origin. It was shown experimentally that several ZDHHC proteins share similar protein substrates. Interestingly, using our phylogenetic classification, we found that these ZDHHC proteins are also classified within the same phylogenetic group. Therefore, it seems that ZDHHC proteins that are evolutionary closely related have overlapping protein substrate specificities, which might provide important indications to study the remaining ZDHHC proteins that have not been characterized yet.

Zusammenfassung

Lysosomen bilden ein wichtiges Zellkompartiment, welches zelluläre Abfallstoffe abbaut und die dabei frei werdenden Fragmente für die Biosynthese neuer Verbindungen von der Zelle verwendet werden können. Die essentielle Bedeutung der Lysosomen - sowohl für die Zelle als auch für den gesamten Organismus - wird durch die Tatsache verdeutlicht, dass Beeinträchtigungen der lysosomalen Funktionen zu schweren metabolischen Erkrankungen, wie zum Beispiel Mucolipidosen, Gaucher Syndrom und Niemann-Pick Syndrom, führen können. Der 'cation-dependent mannose 6-phosphate' Rezeptor erhält die Funktion der Lysosomen aufrecht, indem er neu synthetisierte lysosomale Enzyme vom sekretorischen Transportweg abbringt und sie in die Lysosomen transportiert. Zur Erfüllung dieser Aufgabe bindet der 'cation-dependent mannose 6-phosphate' Rezeptor die lysosomalen Enzyme innerhalb des trans-Golgi Netzwerks und transportiert sie zu den Endosomen, wo er sie freigibt. Anschliessend begibt er sich zurück zum trans-Golgi Netzwerk, um eine neue Runde des Transports zu beginnen. Das korrekte Sortieren des Rezeptors in den Endosomen ist entscheidend, um einen irrtümlichen Transport des Rezeptors in die Lysosomen zu verhindern, wo er abgebaut würde. Interessanterweise konnte in einer früheren Arbeit gezeigt werden, dass die post-translationale Modifikation durch S-Palmitoylierung essentiell für das korrekte Sortieren des Rezeptors in den Endosomen ist. Zudem konnte in der Plasmamembran und in den Endosomen eine Protein-Acyl-Transferase-Aktivität für den 'cation-dependent mannose 6-phosphate' Rezeptor gefunden werden. In dieser Dissertation wurden zwei post-translationale Modifikationen (die S-Palmitoylierung und die Ubiquitinierung) und deren Rollen für das korrekte Sortieren des 'cation-dependent mannose 6-phosphate' Rezeptors in den Endosomen untersucht. Es wurde gezeigt, dass der Rezeptor an seinem

cytosolischen Ende ubiquitiniert wird. Zudem wurde dargestellt Belege gegeben, dass diese Modifikation Teil eines Qualitätskontroll-Mechanismus auf Ebene der Plasmamembran und der Endosomen sein könnte. Des Weiteren wird in dieser Arbeit aufgezeigt, dass die S-Palmitoylierung die Assoziation des Rezeptors mit cholesterin-reichen Membrandomänen ermöglicht, welche sehr wahrscheinlich in frühen Endosomen lokalisiert sind. Diese cholesterin-reichen Membrandomänen werden für den Rücktransport des 'cation-dependent mannose 6-phosphate' Rezeptors von den Endosomen zum trans-Golgi Netzwerk gebraucht. Somit scheint die S-Palmitoylierung ein molekularer Schalter zu sein, welcher die Assoziation des Rezeptors mit cholesterin-reichen Membrandomänen in Endosomen und somit dessen Rücktransport zum trans-Golgi Netzwerk erlaubt. Um das Enzym, welches die S-Palmitoylierung des 'cation-dependent mannose 6-phosphate' Rezeptors katalysiert, identifizieren zu können, wurde eine neue Familie von Protein-Acyl-Transferasen (die ZDHHC Proteinfamilie) untersucht. In der Folge wurde eine neue Phylogenese der ZDHHC Proteinfamilie aufgestellt, die intrazelluläre Lokalisation der einzelnen ZDHHC Proteine bestimmt und deren enzymatische Aktivität für den 'cation-dependent mannose 6-phosphate' Rezeptor analysiert. Als Resultat dieser Dissertation kann ein neues Model für den Transport des 'cation-dependent mannose 6-phosphate' Rezeptors innerhalb der Endosomen aufgestellt werden. Des Weiteren werden eine limitierte Zahl an ZDHHC Proteinen als mutmassliche Protein-Acyl-Transferasen für den 'cation-dependent mannose 6-phosphate' Rezeptor identifiziert. Die phylogenetische Analyse der ZDHHC Proteinfamilie offenbarte zudem Gruppen von ZDHHC Proteinen mit einem nahen evolutionären Ursprung. Es konnte experimentell gezeigt werden, dass mehrere ZDHHC Proteine ähnliche Protein-Substrate besitzen. Interessanterweise waren diese Proteine denn auch unter Anwendung unserer phylogenetischen Klassifikation in der selben

phylogenetischen Gruppe eingeordnet worden. Es scheint deshalb, dass evolutionär nahe miteinander verwandte ZDHHC Proteine überlappende Proteinsubstrat-Spezifitäten besitzen. Dieses Wissen liefert wertvolle Hinweise für die Untersuchung der verbleibenden, noch nicht charakterisierten ZDHHC Proteine.

General introduction

This general introduction is composed of six different parts. The first part briefly introduces the organelles of the secretion/endocytosis pathway. The second part and the third part treat with the transport of biomolecules throughout the secretion/endocytosis pathway. The forth part presents the cation-dependent mannose 6-phosphate receptor which has a central role in this PhD work. Finally, the fifth part and sixth part describe two post-translational modifications (i.e. S-palmitoylation and ubiquitination respectively) mentioned in this work.

Note: the aim of this general introduction is not to summarize all knowledge about the topics presented, but rather to provide the casual biologist with knowledge that is essential for the full understanding of this work and its implications.

Part I: Overview of the organelles of the secretory/endocytic pathway

Foreword

During the late 19th century, early cellular biologists grossly classified intracellular compartments according to technological tools available at the time. However, the picture of intracellular physiology drastically changed since then due to the constant progress of biochemical and optical techniques.

The Golgi apparatus is a good example of this phenomenon. Although it was first observed during the end of the 19th century, the existence of this organelle was debated until the middle of the 20th century, when the emergence of electron microscopy definitively closed the controversy. However, by the end of the 20th century, advances in the field of biochemistry and microscopy obliged scientists to sub-compartmentalize the Golgi apparatus itself in several sub-domains to better reflect newly discovered functions and structural features.

In the following part, I will introduce various cellular compartments relevant to this work according to the “up-to-date” knowledge. Those compartments are the endoplasmic reticulum, the Golgi apparatus, the plasma membrane, the endosomal compartment and the lysosomal compartment. All those compartments are parts of the secretory/endocytic pathway.

The endoplasmic reticulum

The endoplasmic reticulum (ER) is a large cellular compartment which morphologically appears as a network of cisternae and tubules radiating throughout the cell from the nucleus to the plasma membrane (PM).

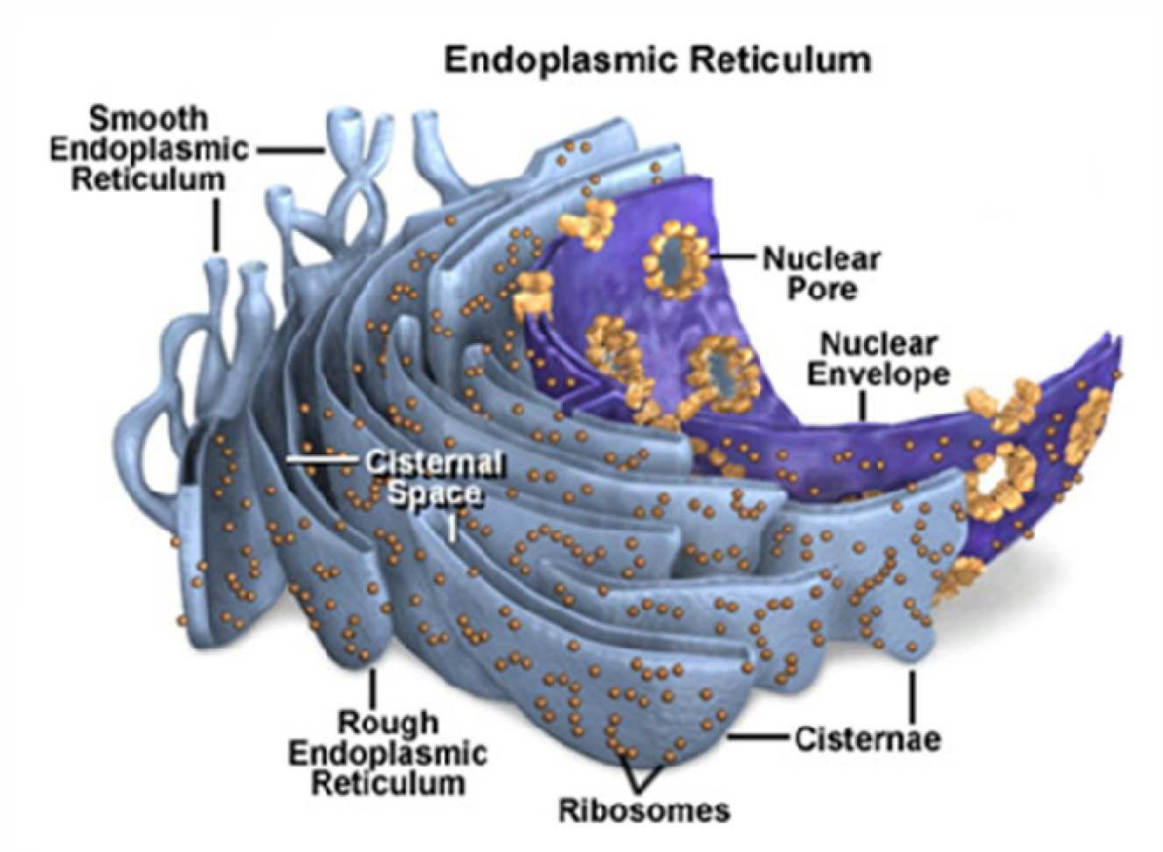

Figure 1: 3D representation of the endoplasmic reticulum and its main sub-domains as indicated (picture from http://www.microscopy.fsu.edu).

The ER is mainly known for its central functions in protein synthesis. Secreted proteins, integral membrane proteins and some peripheral membrane proteins are synthesized on the membranes of the ER, and simultaneously translocated into its lumen for further processing. However, the ER is also involved in cellular metabolism, ionic homeostasis, and provides a structural scaffold to the nucleus.(Sitia and Meldolesi, 1992).

The different functions of the ER are fulfilled by different sub-domains of the ER. The rough ER (Figure 1) is specialized in the synthesis, post-translational modification, and folding of proteins (Kleizen and Braakman, 2004). The smooth ER

(Figure 1) regulates calcium homeostasis (Gorlach *et al.*, 2006), participates in glycogen, lipid and steroid metabolism (van Schaftingen and Gerin, 2002; Fagone and Jackowski, 2009), and also contains several enzymes involved in drug detoxification (Iyanagi, 2007). The nuclear envelope (Figure 1) is yet another sub-domain of the ER, and compartmentalizes the genetic information (Voeltz *et al.*, 2002). Finally, at the level of the transitional ER, newly synthesized proteins can be packaged into transport vesicles to exit the ER (Orci *et al.*, 1991; Hobman *et al.*, 1998). Altogether, the ER is a crucial cellular compartment for being implicated in important cellular functions.

The endoplasmic reticulum Golgi intermediate compartment, Golgi apparatus and trans-Golgi network

After exiting the ER, newly synthesized proteins are first reaching the endoplasmic reticulum Golgi intermediate compartment (ERGIC) before moving further to the Golgi apparatus (GA). The ERGIC appears as a compartment localized in close proximity of the transitional ER (Klumperman *et al.*, 1998). To date, the ERGIC is believed to be mainly involved in the concentration of cargo biomolecules *en route* for the Golgi apparatus, and in the selective retrieval to the ER of ER resident proteins which left the ER by mistake with the bulk flow of newly synthesized proteins (Appenzeller-Herzog and Hauri, 2006). Once exiting the ERGIC, newly synthesized proteins enter the Golgi apparatus. The Golgi apparatus is a cellular compartment which is specialized in the modification of the lipids and the proteins that were previously synthesized in the ER (Kukuruzinska and Lennon, 1998; Sandhoff and Kolter, 2003).

Structurally, the GA is composed of a series of approximately six flattened cisternae which are located in the vicinity of the nucleus (figure 2) (Polishchuk and Mironov, 2004).

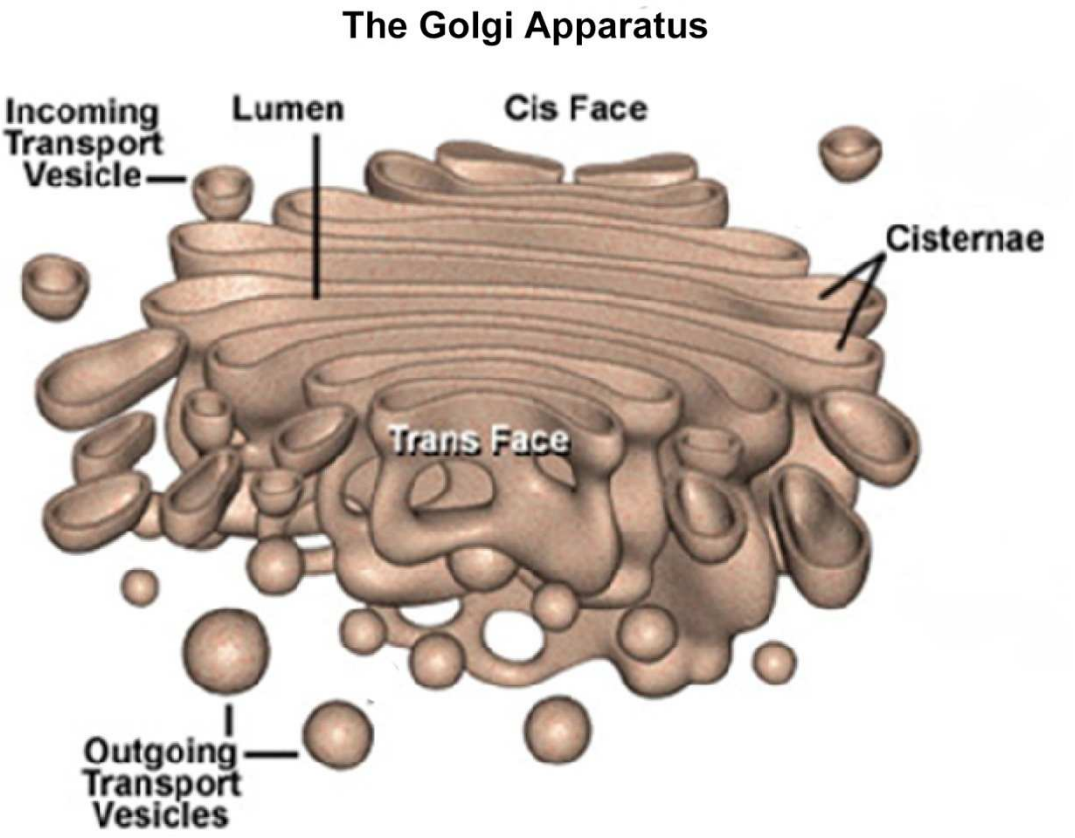

Figure 2: 3D representation of the Golgi apparatus with its main sub-domain as indicated (picture from http://www.microscopy.fsu.edu)

Newly synthesized proteins enter the Golgi apparatus by the cis-Golgi cisternae, and then traffic in a stepwise manner through the different cisternae to ultimately exit the Golgi apparatus by its trans-face (Jackson, 2009). During their trafficking through the Golgi apparatus, proteins acquire various post-translational modifications catalyzed by enzymes localized within the lumen of the cisternae (Rabouille *et al.*, 1995). Similarly to the endoplasmic reticulum that has specialized sub-domains, the different Golgi cisternae contain different sets of enzymes (Rabouille *et al.*, 1995). After leaving the Golgi apparatus, proteins and lipids enter the trans-Golgi network (TGN) which is a compartment specialized into the sorting

of proteins to various intracellular localizations such as the plasma membrane, the endosomes or the lysosomes (De Matteis and Luini, 2008).

Most importantly for this work, the Golgi apparatus is the compartment where the formation of the mannose 6-phosphate residues is catalyzed on the N-glycans of soluble lysosomal enzymes. Those mannose 6-phopshate residues are crucial for the proper sorting of those enzymes to the lysosomal compartment (Braulke and Bonifacino, 2008).

Overall, the GA appears as a cellular compartment involved in the processing of biomolecules previously synthesized within the ER.

The plasma membrane

Upon departure from the GA, the default pathway leads proteins and lipids to the plasma membrane (Figure 3) and to the extracellular milieu (Stevens *et al.*, 1986; Wieland *et al.*, 1987; Denecke *et al.*, 1990; Karrenbauer *et al.*, 1990; Lewis *et al.*, 1990).

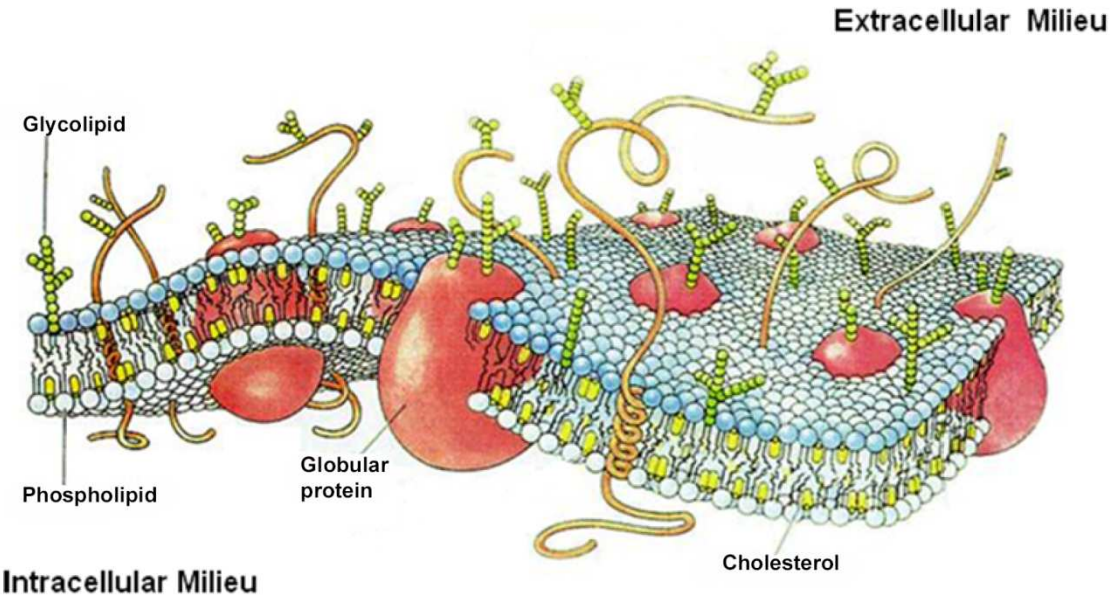

Figure 3: Schematic representation of the plasma membrane and its main components as indicated (picture from http://www.wikivisual.com)

Although the plasma membrane is not a cellular compartment *per se*, it is a crucial cellular domain. It has functions in nearly all aspects of cellular biology. For example, the plasma membrane has structural and metabolic functions (Daniel, 2004), and is important for ionic homeostasis (Rabouille *et al.*, 1995) as well as cellular division (Haugh, 2002) and motility (Pollard and Borisy, 2003). The loss of integrity of the plasma membrane mostly leads to cell death.

Various proteins and lipids synthesized along the secretory pathway are embedded within the plasma membrane (Figure 3). The plasma membrane is a very dynamic cellular structure which has different sub-domains fulfilling various functions (e.g. internalization, cell signaling, cell-cell interactions…) (Lajoie *et al.*, 2009).

The cell can dynamically modulate the composition of the plasma membrane by removing lipids and proteins through internalization. After internalization at the plasma membrane, proteins and lipids are mainly delivered to the endosomal compartment (Riezman *et al.*, 1997).

The endosomal compartment

The endosomal compartment is a dynamic compartment which is in constant exchange with the plasma membrane through extensive vesicular trafficking. Upon internalization at the plasma membrane, proteins and lipids reach the endosomal compartment that sorts them to various cellular compartments such as the lysosomal compartment, the Golgi apparatus or the plasma membrane (Seaman, 2008). Consequently, the endosomal compartment has an extensive control over many cellular functions through its ability to actively recycle proteins back to the plasma membrane or to terminate them by targeting them for degradation in lysosomes (Perret *et al.*, 2005).

The endosomal compartment displays four main different functional sub-compartments: the recycling endosomes, the multi-vesicular bodies (MVBs), the tubular endosomal network and the late endosomes (Marsh *et al.*, 1986). Recycling endosomes sustain the recycling of proteins back to the plasma membrane (van Ijzendoorn, 2006). The tubular endosomal network participates in the transport of biomolecules from the endosomes to the Golgi apparatus.(Bonifacino and Hurley, 2008). Finally, the MVBs form an endosomal sub-domain filled with intra-luminal vesicles (ILVs) containing down-regulated proteins (Piper and Katzmann, 2007). Once MVBs are loaded with cargo biomolecules, they mature to become late endosomes, or fuse with late endosomes (Falguieres *et al.*, 2009). The late endosomes form a specialized endosomal compartment which has close interactions with the lysosomes to allow the delivery and subsequent degradation of selected cargo biomolecules (Luzio *et al.*, 2007).

Altogether, the endosomal compartment appears as a trafficking hub which has an extensive exchange with multiple cellular compartments.

The lysosomal compartment

The lysosomal compartment is involved in the degradation of cellular waste products or biomolecules (fatty acids, carbohydrates or proteins) into simple compounds which can be subsequently recycled for the biosynthesis of new components (De Duve and Wattiaux, 1966). To fulfill its function, the lysosomal compartment is equipped with a broad spectrum of proteins displaying hydrolytic activities (De Duve *et al.*, 1955). Most relevant to this work, a large subset of lysosomal enzymes relies on mannose 6-phosphate residues to be delivered successfully to the lysosomal compartment by the mannose 6-phosphate receptors. Therefore, the ability of the mannose 6-phosphate receptors to properly cycle

throughout the cell is crucial for the biogenesis of the lysosomal compartment (Kornfeld and Mellman, 1989).

Part II: The molecular machinery involved in the transport of biomolecules between the organelles of the secretory/endocytic pathway.

Foreword

As we saw in the previous part, the eukaryotic cell contains various organelles that are crucial for its survival. In order to maintain those organelles in a functional state, biomolecules need to be properly sorted to their correct intracellular localization. The intracellular transport of biomolecules is mainly sustained by transport vesicles which bud from a donor compartment to subsequently fuse with an acceptor compartment. However, some other mechanisms to transport biomolecules throughout the cell also exist. In this part, the main protein families sustaining the intracellular trafficking of biomolecules are presented. A detailed description of the individual members of those protein families is given in another part (i.e. part III: "intracellular routes"), when the different intracellular trafficking pathways are discussed.

The Ras super-family

Proteins classified into the rat sarcoma protein super-family (Ras-like proteins) are small GTPases bearing a conserved "G box" which confers them the biochemical ability to bind GTP and to hydrolyze it into GDP. (Dever *et al.*, 1987). The binding of a GTP molecule on the "G box" of a Ras-like GTPase leads to a conformal change. This conformational change allows the Ras-like GTPase to interact with effector proteins. The interaction of the Ras-like GTPase with its effector proteins is terminated by the hydrolysis of the GTP molecule into a GDP molecule. Thus, the GTP-bound form of the Ras-like GTPase is routinely called the "active state" and the GDP-bound form the "inactive state" (Colicelli, 2004).

The activation of Ras-like GTPases is mainly controlled by three protein families which are the guanine nucleotide exchange factor (GEF) protein family, the GTPase activating protein (GAP) family and the guanine nucleotide dissociation inhibitor (GDI) protein family.

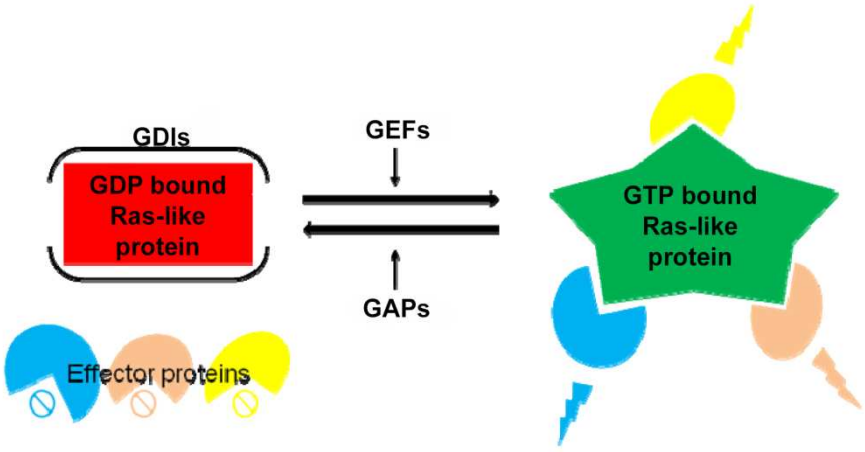

Figure 4: Symbolic representation of Ras-like GTPases, their regulators and effectors. In red is represented the inactive Ras-like GTPase state which fails to interact with its effectors and is stabilized by Guanine nucleotide dissociation inhibitors (GDIs). In green is represented the active Ras-like GTPase state which interacts with its effectors. Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) promote the conversion of the Ras-like GTPase from one state to the other.

GEF proteins promote the activation of Ras-like GTPases by catalyzing the dissociation of GDP molecules from Ras-like GTPases, whereas GAP proteins enhance the intrinsic GTPase activity of Ras-like GTPases (thus promoting their inactivation). Finally, GDI proteins prevent the activation of Ras-like GTPases by stabilizing their GDP-bound form (figure 4) (Colicelli, 2004).

The Ras-like GTPases super-family is composed of hundred seventy members which are further sub-classified into four groups (i.e. the Ras, Rho, Rab and Arf protein families) (Colicelli, 2004). Most relevant for this work, members of the Arf and Rab family (thirty and seventy-one members respectively) are implicated in the intracellular trafficking of biomolecules by promoting the remodeling of membrane sub-domains, the formation of transport vesicles, the motility of those transport vesicles and their fusion with target compartments (Wennerberg *et al.*, 2005;

Gillingham and Munro, 2007). Upon activation, Arf and Rab GTPases are binding intracellular membranes on which they will promote the formation of specialized membrane sub-domains, and/or induce the formation of transport vesicles through the recruitment of various effector proteins with scaffolding and enzymatic abilities (Behnia and Munro, 2005). Thus, Arf and Rab GTPases are considered as master-key players for the engineering and maintenance of cellular compartments.

The best understood Ras-like GTPase is most probably one found on early endosomes: Rab5. Upon activation, Rab5 associates with endosomal membranes where it recruits a phosphatidylinositol kinase (VPS34) which enriches the endosomal membranes with phosphatidylinositol 3-phosphate (Christoforidis *et al.*, 1999). Subsequently, endosomal membranes can recruit EEA1 (Merithew *et al.*, 2003) and Rabenosyn5 (Nielsen *et al.*, 2000), which are two factors required for the homotypic fusion of endosomal membranes and the fusion of transport vesicles with the endosomal compartment. Additionally, Rab5 might be able to mediate the formation of transport vesicles through the action of its effector Rabaptin-5 (Mattera *et al.*, 2003). This brief example shows how Ras-like GTPases can engineer specific membrane sub-domains and promote vesicular trafficking.

Sorting nexin family

Sorting nexin (SNX) family is a family of peripheral membrane proteins (approximately twenty-eight members in mammals) that have been grouped together based upon the presence of a phox (PX) domain in their sequences. This PX domain allows SNXs to bind to different phosphoinositides present on the membranes of intracellular compartments (Carlton *et al.*, 2005). Experimental data showed that some members of the SNX family are sustaining the transport of

several biomolecules throughout the cell (Bonangelino *et al.*, 2002; Worby and Dixon, 2002).

The transport of biomolecules mediated by SNXs does not seem to involve transport vesicles, but rather large tubular-shaped transport carriers (Bonifacino and Hurley, 2008). Still little is known about the precise mechanisms underlining the transport of biomolecules mediated by SNXs. However, it has been observed that some SNXs associate into a protein complex to fulfill their trafficking function (Collins, 2008). The SNXs-based protein complex that is best characterized is the retromer. The retromer is formed by the association of SNX1 and SNX2 with the three other proteins VPS29, VPS35 and VPS26 (Figure 5) (Seaman, 2005). Within the retromer, SNX1 and SNX2 are believed to have a structural role, whereas the VPS29-35-26 sub-complex recognizes and captures cargo proteins. (Figure 5).

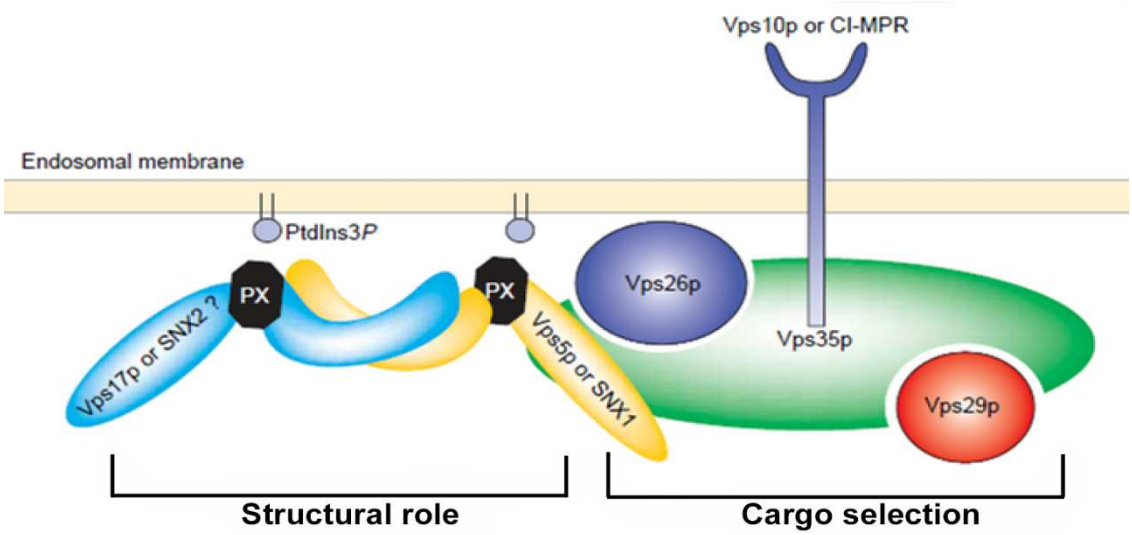

Figure 5: Representation of the SNX1, SNX2, VPS26, Vps35 and Vps29 assemble into a protein complex called “the retromer”. The phox domain (PX) of SNX1 and SNX2 allow the retromer to recognize and assemble on endosomal membrane where it can promote the retrieving of cargo proteins (Vps10 or CI-MPR) to the Golgi apparatus. PtdIns3P: phosphatidylinositol 3-phosphate. Picture from (Seaman, 2005).

Adaptins

Adaptins are protein complexes (e.g. AP-1, AP-2, Sec23–Sec24) or single proteins (e.g. GGAs, stonin, HRS) that are effectors of Ras-like GTPases (Boehm and

Bonifacino, 2001). Consequently, adaptins are recruited onto cellular membranes by activated Ras-like GTPases. Once recruited onto cellular membranes, adaptins can simultaneously bind cargo proteins and recruit coat proteins from the cytosol. Coat proteins are usually forming the structural scaffold of transport vesicles. Therefore, the interaction of adaptins with coat proteins usually promotes the formation of transport vesicles. By recruiting cargo proteins and promoting the formation of transport vesicles simultaneously, adaptins mediate the formation of transport vesicles enriched with cargo proteins. It is important to underline that fact that the affinity of adaptins to cargo proteins is highly specific. Therefore, each adaptin can only recruit a defined set of cargo proteins (Robinson, 1992; Kirchhausen *et al.*, 1997). Consequently, the cargo of a transport vesicle directly depends on the adaptins it contains.

Coat proteins

When recruited onto the cellular membranes, coat proteins (e.g. Clathrin, Sec13–Sec31) polymerize into a protein lattice (Lippincott-Schwartz and Liu, 2006; Young, 2007). With the action of proteins specialized in the curving of cellular membranes (e.g. epsin (Ford *et al.*, 2002) and endophilins (Farsad *et al.*, 2001)), a bud can emerge from a cellular membrane coated with a coat protein lattice (Figure 6). When the bud grows to reach a size defined by the intrinsic structural properties of the coat proteins forming the protein lattice (Bi *et al.*, 2002), it can be detached from the donor membrane to form a new transport vesicle. The detachment of the bud from the donor membrane requires the action of proteins specialized in fission of cellular membranes such as amphiphysins (Wigge and McMahon, 1998) and dynamins (Praefcke and McMahon, 2004) (Figure 6).

Ras-like GTPase/adaptin/coat proteins complex

The formation of a new transport vesicle is sustained by the tight interplay between Ras-like GTPases, adaptins, cargo proteins and coat proteins (Figure 6). Figure 7 shows some example of coat/adaptin/Ras-like GTPase complexes already found to be involved in the formation of transport vesicles.

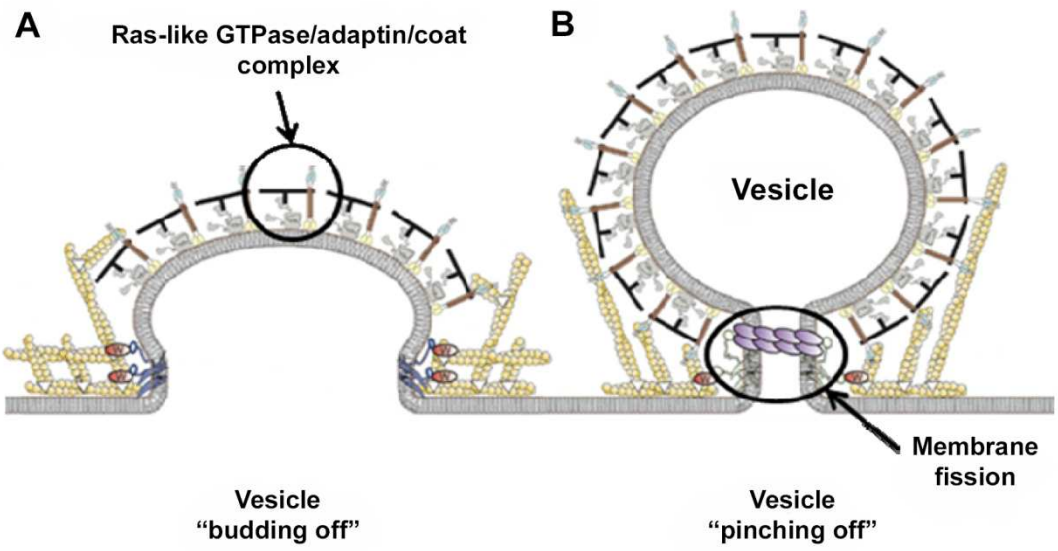

Figure 6: Schematic representation of the "budding off" and the "pinching off" of a new transport vesicle. (A) Ras-like GTPase/adaptin/coat protein complexes assemble onto a biological membrane and promote the "budding off" of a new transport vesicle. (B) When the membrane bud reach a defined size, the release of a new transport vesicle is generated by the activity of proteins mediating membrane fission. Adapted from (Brett and Traub, 2006)

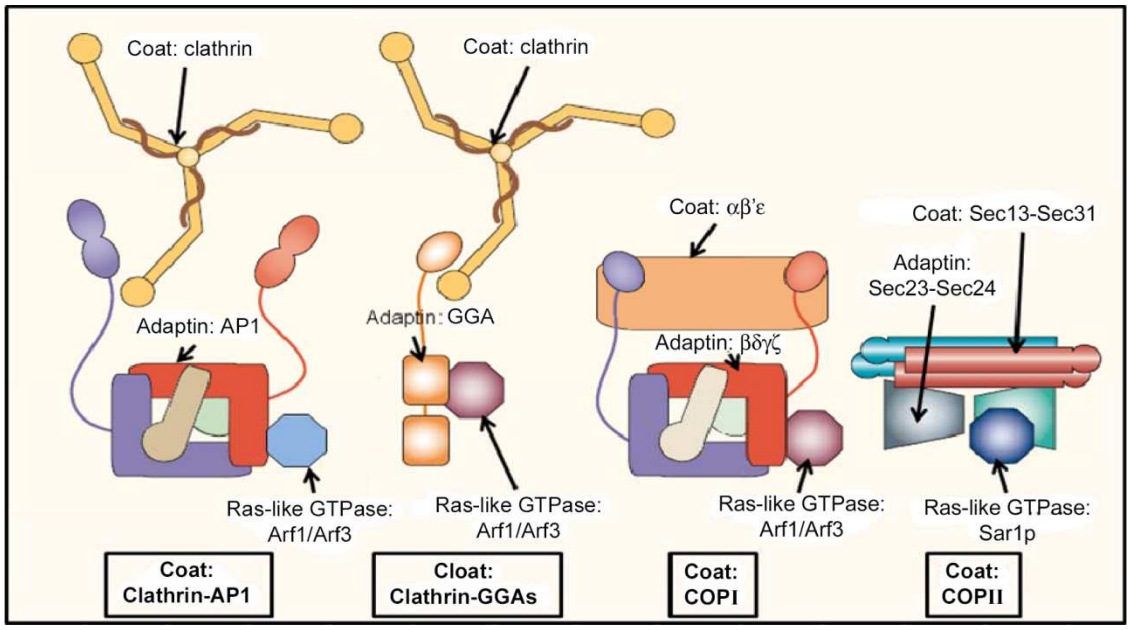

Figure 7: Schematic representation of Ras-like GTPase/adaptin/coat protein complexes involved in the formation of trafficking vesicles. Adapted from (Bonifacino and Traub, 2003).

After their formation, transport vesicles must travel throughout the cell and specifically fuse with a target compartment. Two groups of proteins are responsible for the proper docking and fusion of transport vesicles with their target compartments: tether proteins and SNARE proteins.

Tether proteins

Tether proteins are proteins which are required for in the initial docking of a transport vesicle with a target compartment. The exact mechanism by which tether proteins mediate the docking of transport vesicles is not clearly understood yet (Sztul and Lupashin, 2006). Tether proteins are similar to each other only in their overall structural aspect. Therefore, they are classified in two groups according their structural properties: long coiled-coil proteins (e.g. giantin, p115, EEA1…) or multi-subunit protein complexes (e.g. COG complex, Exocyst, Dsl…) (Sztul and Lupashin 2006). Tether proteins localize on cellular compartments and on transport vesicles on which they are often stabilized by Ras-like GTPases (Short *et al.*, 2005; Markgraf *et al.*, 2007). The localization of the various tethers in the cellular environment is depicted in figure 8. The fact that different tether proteins are recruited onto precisely defined sets of transport vesicles and cellular compartments makes it highly probable that they play a central role in the specificity of the intracellular trafficking of transport vesicles.

The vesicle docking activity of tether proteins has been shown to be an essential prerequisite to the fusion of a transport vesicle with a cellular compartment (Cai *et al.*, 2007a). However, the fusion itself is mediated by another protein family: the SNARE protein family.

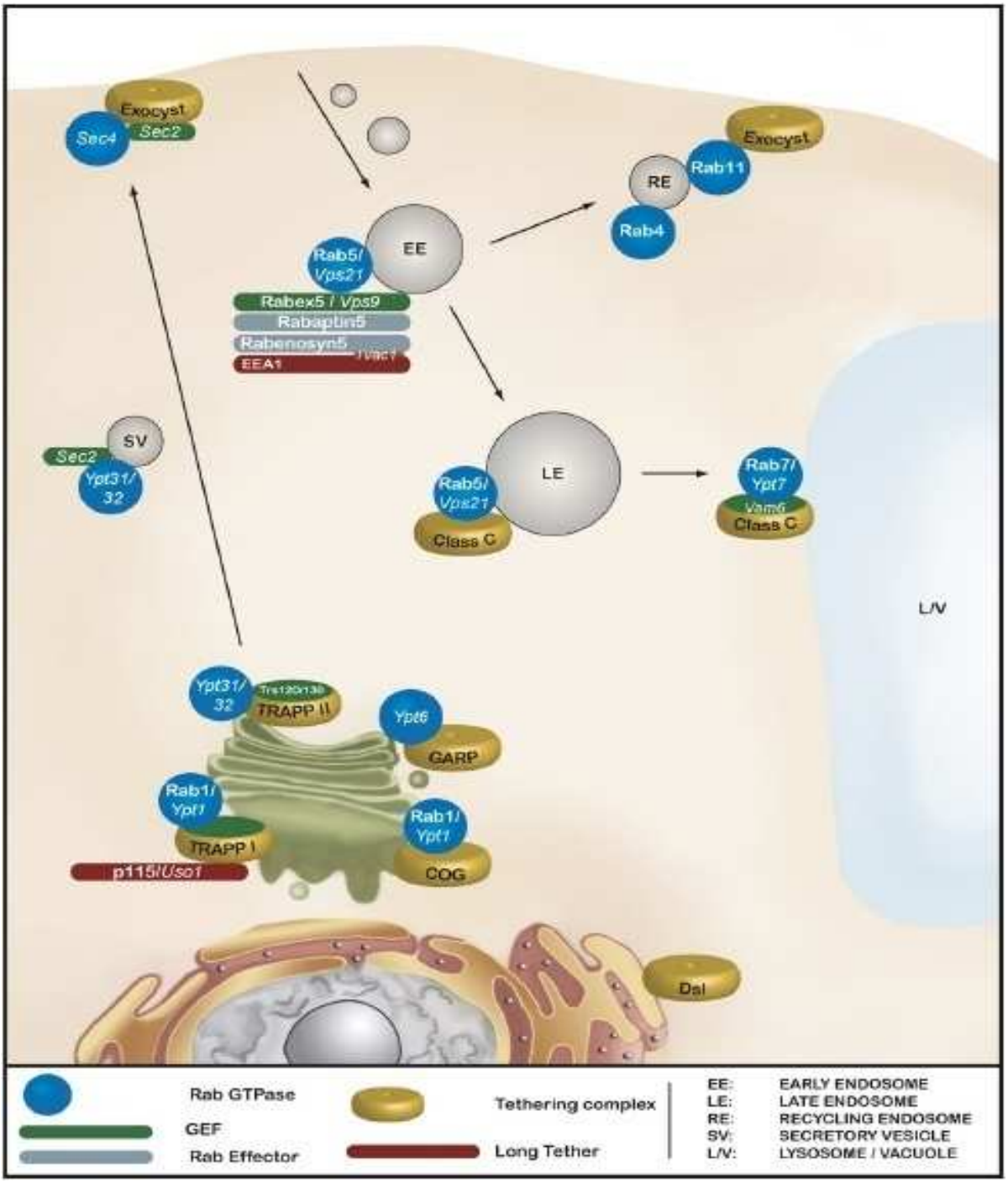

Figure 8: Schematic representation of known tethering complexes associated with their respective intracellular membranes. The Ras-like GTPases (here exclusively Rabs) which mediate the recruitment of the discrete tether complexes is also represented. Picture from (Markgraf *et al.*, 2007).

The SNARE protein family

The SNARE protein family is a family of membrane proteins which mediates the fusion of transport vesicles with various cellular compartments. SNARE proteins share a distinctive protein domain of sixty amino acids (the SNARE motif) which is required for their fusion activity. Usually, each SNARE protein associates preferentially with transport vesicles (v-SNARE) or with cellular compartments (t-SNARE) (Duman and Forte, 2003). Thus, when a transport vesicle is stabilized in the close vicinity of a cellular compartment by the action of tether proteins, a single v-SNARE and three t-SNAREs are able to form a tight complex (Antonin *et al.*, 2002). In a mechanism very similar to the one of a zipper, the v-SNARE/t-SNARE complex brings the transport vesicle closer to the target compartment, until the transport vesicle fuses with the target compartment (Sudhof and Rothman, 2009).

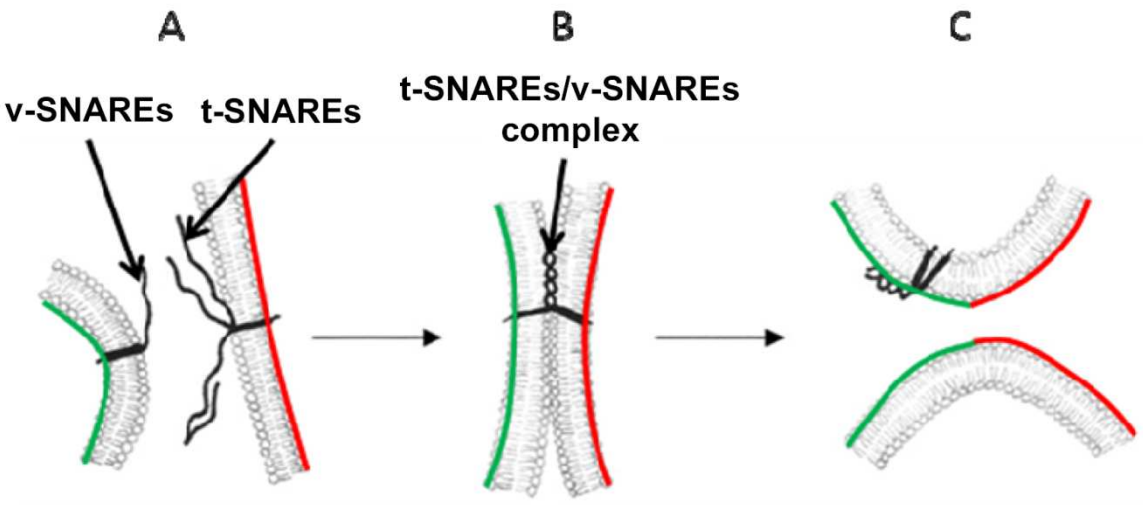

Figure 9: Schematic representation of the fusion of the membrane of a transport vesicle (in green) and the membrane of an acceptor compartment (in red). (A) When in close proximity, the v-SNARE proteins present on the transport vesicle and the t-SNARE proteins present on the acceptor compartment can interact to form a t-SNAREs/v-SNAREs complex (represented in B). (B) The formation of the t-SNAREs/v-SNAREs complex leads to the apposition of the transport vesicle onto the target compartment. (C) Ultimately, the membranes of the transport vesicle and the target compartment fuse. The content of the transport vesicle is released into the target compartment. Adapted from (Duman and Forte, 2003).

Similarly to tether proteins, the different SNARE proteins display different intracellular localizations. In addition, defined subsets of v-SNARE proteins exhibit

affinity to restricted subsets of t-SNAREs (Jahn and Sudhof, 1999). Therefore, a transport vesicle containing certain v-SNARE can only fuse with a cellular comportment displaying certain t-SNARE. Consequently, the SNARE system also participates to the specificity of the trafficking of transport vesicles throughout the cell. Various mammalian SNARE proteins and the trafficking routes on which they are functioning are depicted in the Figure 10.

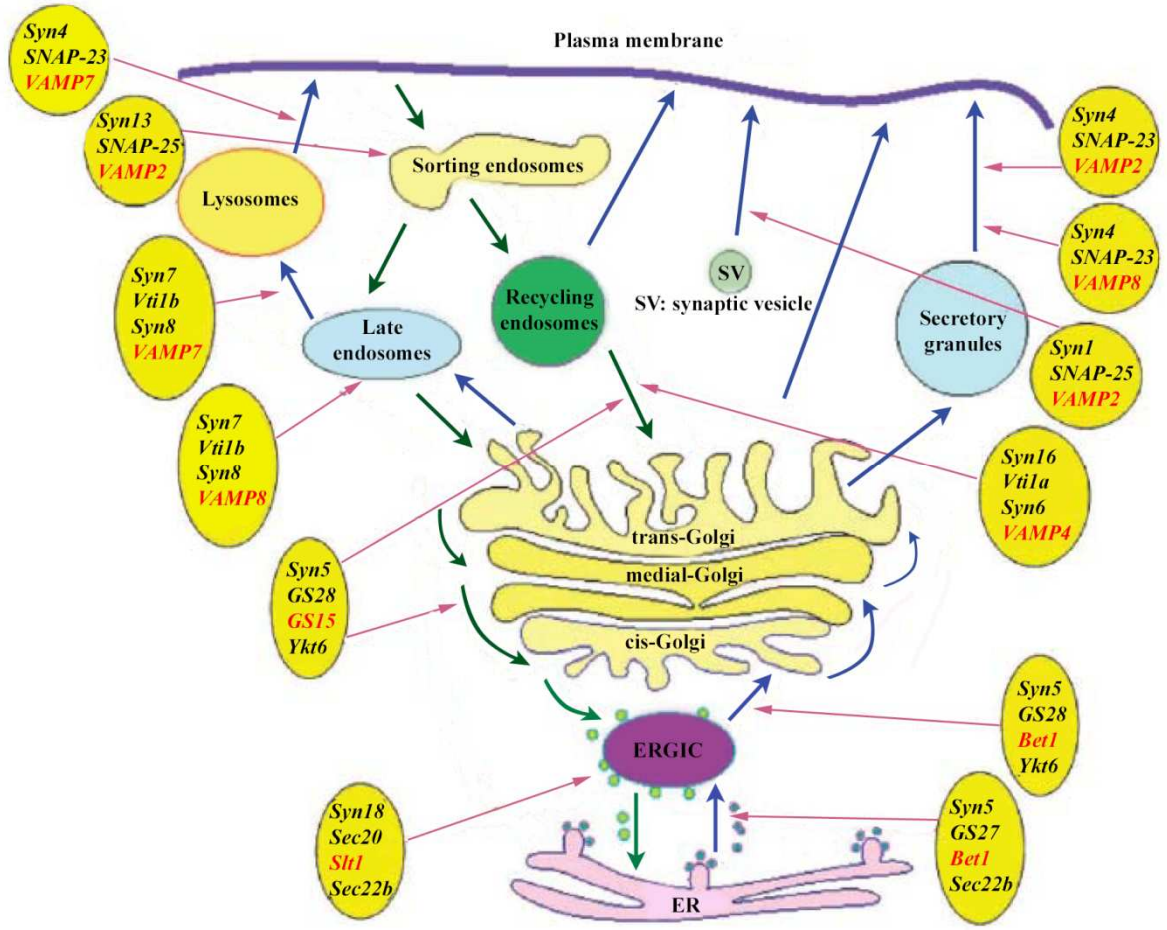

Figure 10: Schematic representation of the secretory/endocytic pathway. The SNARE proteins known to be involved in the different trafficking routes are indicated in yellow circles. Picture from (Hong, 2005)

Upon fusion of a transport vesicle with a cellular compartment, the cargo biomolecules contained into the transport vesicle are delivered into a new cellular compartment. All the specific interactions between the groups of proteins presented above ensure the specificity and accuracy of the trafficking of transport vesicles throughout the cell.

Part III: Intracellular transport routes of the secretory/endocytic pathway

Foreword

As discussed above, the intracellular transport of biomolecules is sustained by different families of protein with specific functions. Each of those families (Ras-like GTPases, adaptins, etc…) consists of many members, and each of those members is often operating on a defined subset of intracellular transport routes. In the following section, the intracellular transport routes of the secretory/endocytic pathway will be presented.

The trafficking pathway from the ER to the Golgi apparatus

The ER to Golgi transport route (figure 11) is sustained by transport vesicles coated with the coat protein COPII. All proteins synthesized on the ER membrane and *en route* for subsequent cellular compartments are believed to use this trafficking pathway. In yeast, the formation of COPII-coated transport vesicles is initiated by the GEF Sec12 which activates and recruits the Ras-like GTPase Sar1p onto ER membranes (Aridor *et al.*, 2001; Weissman *et al.*, 2001). Once stabilized onto the ER membrane, Sar1p recruits a hetero-dimeric adaptin Sec23-Sec24 complex (Bi *et al.*, 2002). Sec23 and Sec24 are structurally similar proteins that have several isoforms. The functional specificities of the different isoforms of Sec23 and Sec24 are not known yet.

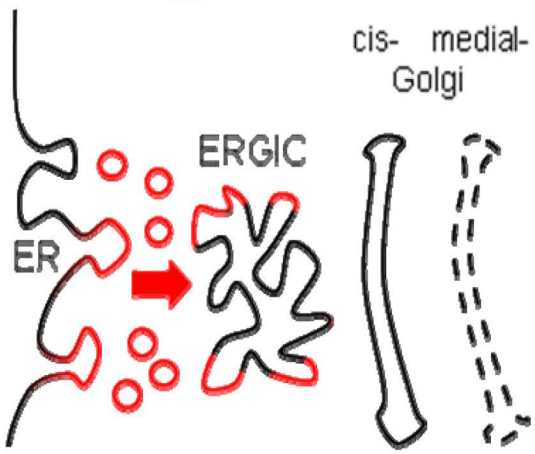

Figure 11: Schematic representation of the ER and Golgi apparatus. The structures involved in ER to Golgi trafficking are represented in red

Sec24 has been found to fulfill many of the adaptin functions of the Sec23-Sec24 complex. Sec24 can recruit cargo proteins bearing an ER export signal (Nishimura *et al.*, 1999) as well as the coat protein complex Sec13-Sec31 (Bickford *et al.*, 2004; Fath *et al.*, 2007). Sec24 also recruits the SNARE proteins Bet1 and Sed5 (Mossessova *et al.*, 2003). Sec22 and Bos1 are two other SNARE proteins that are required for the transport of biomolecules from the ER to the Golgi apparatus (Cao and Barlowe, 2000).

The other subunit of the Sec23-Sec24 complex Sec23 is responsible for the binding of the Sec23-Sec24 complex to the Sar1p GTPase, and also recruits the tether complex TRAPPI (Cai *et al.*, 2007b). P115, yet another tether protein, is recruited on COPII-coated transport vesicles and on Golgi membranes by the small GTPase Rab1, and has been found to be essential for the transport of biomolecules from the ER to Golgi apparatus (Cao *et al.*, 1998).

The trafficking pathways from the Golgi apparatus to the ER and intra-Golgi apparatus

The transport of biomolecules between the different cisternae of the Golgi apparatus and from the Golgi apparatus to the ER is sustained by transport vesicles coated with the coat protein COPI (Figure 12). The generation of a COPI-coated transport vesicle starts with the recruitment onto Golgi membranes of the small GTPase Arf1 (Donaldson *et al.*, 1992; Palmer *et al.*, 1993). The activated Arf1 GTPase

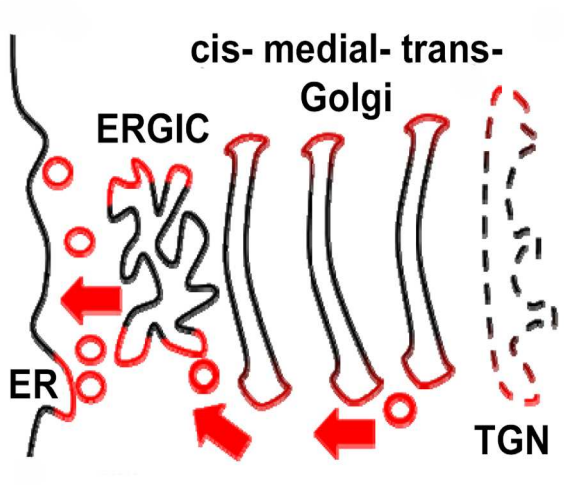

Figure 12: Schematic representation of the ER and Golgi apparatus. The structures involved in intra-Golgi and Golgi to ER trafficking are represented in red

recruits a protein complex called coatomer (composed of α-, β-, γ-, δ-, ε-, ζ- and β'-COPI subunits) onto Golgi membranes. The coatomer have the functions of both an adaptin and a coat protein (Bethune *et al.*, 2006). Phylogenetic studies showed extensive similarities between the αεβ'-COPI protein sub-complex and other coat proteins, as well as between βγδζ-COPI protein sub-complex and other adaptins (Schledzewski *et al.*, 1999). The successful recruitment of the coatomer onto Golgi membranes also depends on the action of the integral membrane protein p24 which recruits the γ-COPI subunit (Harter *et al.*, 1996; Bremser *et al.*, 1999). Once assembled onto the Golgi membranes, the Arf1/p24/coatomer complex recruits integral membrane cargo proteins having a C-terminal Lys-Lys-X-X (KKXX) and Lys-X-Lys-X-X (KXKXX) protein motif where X is any amino acid (Jackson *et al.*, 1990; Harter and Wieland, 1998; Eugster *et al.*, 2004). Soluble cargo proteins bearing a Lys-Asp-Glu-Leu (KDEL) protein motif are also incorporated into COPI-coated transport vesicles by the KDEL receptor ERD2 which has a C-terminal KKXX protein motif itself (Lewis *et al.*, 1990; Majoul *et al.*, 1998).

Several tether proteins and SNARE proteins are involved in the trafficking of COPI-coated transport vesicles. The tether protein giantin is associated with some COPI vesicles and can interact with two other tether proteins localized on cis-Golgi membranes (p115 and GM130) (Short *et al.*, 2005) to mediate the docking of COPI transport vesicles on cis-Golgi membranes. Other tether proteins (i.e. CASP and golgin-84) are associated with another population of COPI coated vesicles which is specialized in the transport of biomolecules between the different cisternae of the Golgi apparatus (Malsam *et al.*, 2005). Finally, the Dsl tethering complex has been shown to be crucial for the proper docking of COPI vesicles to the ER membranes (Andag *et al.*, 2001; Vanrheenen *et al.*, 2001). Thus, discrete COPI vesicle

populations are decorated with different tether proteins, therefore allowing their specific targeting to defined compartments.

Similarly, several pieces of evidence show that the SNARE proteins GS15, syntaxin5, Ykt6 and GS28 are required for the vesicular trafficking of biomolecules between the different cisternae of the Golgi apparatus (Parlati *et al.*, 2002), whereas the SNARE proteins Sec20, Syn18, Use1 and Sec22 are sustaining the fusion of COPI-coated transport vesicles with the ER (Burri *et al.*, 2003; Dilcher *et al.*, 2003).

The trafficking pathways from the TGN to the plasma membrane

Unlike for the transport routes between the Golgi apparatus and the ER, the transport route from the TGN to the plasma membrane does not seem to be sustained by transport vesicles. Instead, research on the transport of biomolecules from the TGN to the plasma membrane revealed the existence of large tubular-shaped carriers (Figure 13). This research also highlighted the importance of membrane fission events for this transport route (Cooper *et al.*, 1990).

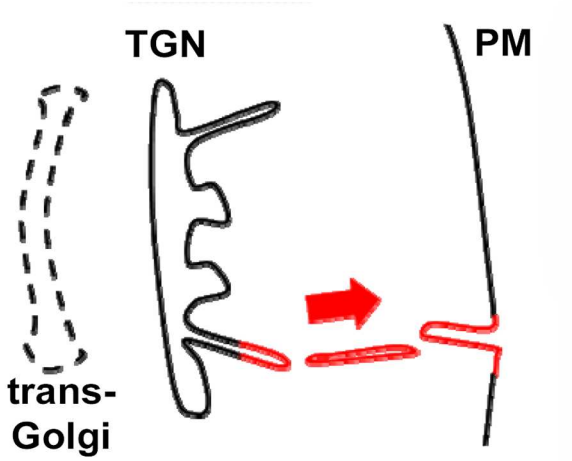

Figure 13: Schematic representation of the Golgi apparatus and the plasma membrane. The structures involved in TGN to PM trafficking are represented in red

Cargo biomolecules *en route* for the plasma membrane accumulate in large tubular-shaped protrusions emerging from the TGN just beyond the trans-cisternae of the Golgi apparatus. Once formed, those large tubular-shaped protrusions detach from the TGN to form large tubular-shaped carriers that are transported to the plasma membrane in a microtubule/kinesin dependent manner (Polishchuk *et al.*, 2003). The mechanisms sustaining the detachment of large tubular-shaped protrusions

from the TGN is not well understood. However, the protein kinase D (PKD) is believed to be a key player of this mechanism because the expression of an inactive mutant form of this protein in HeLa cells results in the stabilization of the large tubular-shaped protrusions emerging from the TGN (Liljedahl *et al.*, 2001). Although no mechanisms are known to selectively incorporate cargo biomolecules in the tubular-shaped protrusions emerging from the TGN, there is evidence that different isoforms of PKD (i.e. PKD1, PKD2, PKD3) are implicated in the transport from the TGN to the plasma membrane of different sets of cargo biomolecules (Yeaman *et al.*, 2004). This fact suggests the existence of different populations of large tubular-shaped carrier. PKD isoforms themselves seems to be regulated by the protein kinase Cη (PKCη) (Diaz Anel and Malhotra, 2005) and by the presence of di-acylglycerol on the membrane of the TGN (Baron and Malhotra, 2002). Blocking the activity of PKCη or disturbing di-acylglycerol homeostasis abolishes the transport of molecules from the TGN to the plasma membrane.

The trafficking pathways from the plasma membrane to the endosomal compartment

Several mechanisms are involved in the transport of biomolecules from the plasma membrane to the endosomal compartment. They are classified in two groups which are the clathrin-dependent endocytosis and the clathrin-independent endocytosis. Clathrin-dependent endocytosis (Figure 14) is believed to be responsible for the majority of the internalization events occurring at the plasma membrane. The clathrin-dependent endocytosis involves the generation of transport vesicles coated with the coat protein clathrin. The generation of clathrin-coated transport vesicles is very similar to the generation of COPI-coated or COPII-coated transport vesicles previously described.

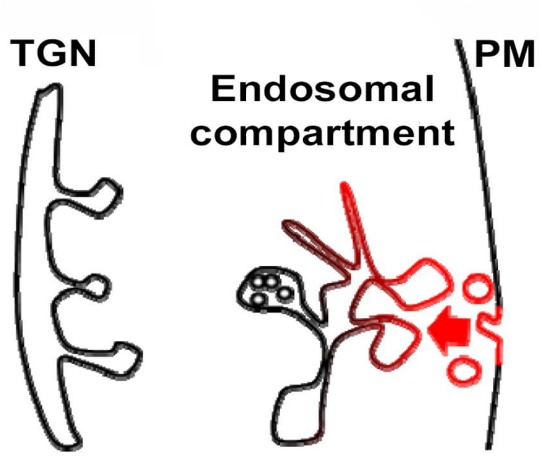

Figure 14: Schematic representation of the Golgi apparatus, the PM and the endosomal compartment. The structures involved in PM to endosomal compartment trafficking are represented in red.

Once activated, the Ras-like GTPase Arf6 binds the plasma membrane and enriches it with phosphatidylinositol 4,5-biphosphate (Krauss *et al.*, 2003; Galandrini *et al.*, 2005) which is a lipid that is required for the association of the clathrin-dependent endocytosis machinery with the plasma membrane. Arf6 also recruits adaptins such has the protein complex AP-2 (Paleotti *et al.*, 2005) or the monomeric β-arrestin (Claing *et al.*, 2001). Other small monomeric adaptins are also involved into clathrin-dependent endocytosis (e.g. Dab2, numb, AP180/CALM, epsin, Eps15 and HIP1/R) (Ungewickell and Hinrichsen, 2007). Once recruited on the plasma membrane, those adaptins recruit various cargo proteins based on their affinity for defined protein motifs. For example, the AP-2 complex binds Tyr-X-X-θ (YXXθ) protein motifs where X is any amino acid and θ is a hydrophobic amino acid. AP-2 also binds [Asp/Glu]-X-X-X-Leu-[Leu/Ile] ([DE]XXXL[LI]) protein motifs as well as Phe-X-Asn-Pro-X-Tyr (FXNPXY) protein motifs. Similarly, the adaptins Dab2 and Arh can bind FXNPXY protein motifs. Finally, the adaptins epsin and Eps15 can bind ubiquitinated cargo proteins, and β-arrestins can bind activated G-protein-coupled receptors. In addition to their ability to bind cargo proteins, all adaptins (except epsin and Eps15) can also bind the coat protein clathrin, therefore sustaining the formation of clathrin-coated buds. The emergence and the growth of clathrin coated buds seem to be supported by tight interactions between the clathrin coat machinery and the actin cytoskeleton (Engqvist-Goldstein and Drubin, 2003). Once a clathrin coated bud is filled with cargo proteins, a new clathrin coated transport

vesicle can be released by the action of the large GTPase dynamin which is specialized in the fission of cellular membrane (Roux *et al.*, 2006). Clathrin-coated transport vesicles are transported throughout the cell onto actin filaments by the action of motor proteins (Spudich *et al.*, 2007).

As previously stated, clathrin-independent internalization mechanisms also exist but our understanding of them is quite limited and sometimes controversial. Those clathrin-independent internalization mechanisms include the caveolin-dependent internalization, the GPI-enriched endosomal compartments pathway, the circular dorsal ruffle -dependent pathway, the flotillin-1-dependent pathway and the dynamin/RhoA/cortactin-dependent pathway (Gong *et al.*, 2008)

The trafficking pathways from the TGN to the endosomal compartment

From the TGN, proteins can also be transported to the endosomal compartment (Figure 15). This transport route is sustained by clathrin-coated transport vesicles. The formation of clathrin-coated transport vesicles at the level of the TGN is initiated by the Ras-like GTPases Arf1 and Arf3 which recruit several adaptins onto the TGN membranes(i.e. AP-1 (Zhu *et al.*, 1998), AP-3 (Ooi *et al.*, 1998), AP-4 (Boehm *et al.*, 2001) and GGAs (Dell'Angelica *et al.*, 2000). Those multiple adaptins bind similar protein motifs for cargo recognition. AP-1, AP-3 and AP-4 bind YXXθ and [DE]XXXL[LI] protein motifs whereas GGAs bind Asp-X-X-Leu-Leu (DXXLL) protein motifs where X is any amino acid. All those adaptins (expect AP-4) can bind

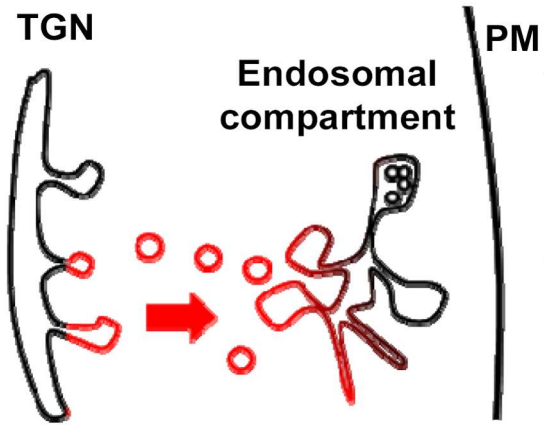

Figure 15: Schematic representation of the Golgi apparatus, the PM and the endosomal compartment. The structures involved in TGN to endosomal compartment trafficking are represented in red.

the coat protein clathrin, and thus promote the formation of clathrin-coated buds. Clathrin coated buds can be separated from the TGN membrane by the combined action of the actin cytoskeleton and dynamin-2 to generate new clathrin coated vesicles (Cao *et al.*, 2005). In many aspects, the mechanisms driving the formation of clathrin-coated transport vesicles at the TGN and at the plasma membrane (presented in the chapter "The trafficking pathway from the plasma membrane to the endosomal compartment") are very similar.

The trafficking pathways from the endosomal compartment to the lysosomal compartment

It is believed that the main entry to the lysosomal compartment is through the endosomes (Figure 16). One way for endosomal proteins to traffic to the lysosomal compartment is to be sorted into the intra-lumenal vesicles (ILVs) of the multi-vesicular bodies (MVBs). It is important to notice that, unlike other known vesicular trafficking pathways, this particular pathway involves the incorporation of the cargo proteins into vesicles that bud into an organelle, rather than into the cytosol. This requires a completely different machinery for the sorting of cargo proteins as well as for the generation of the transport vesicles.

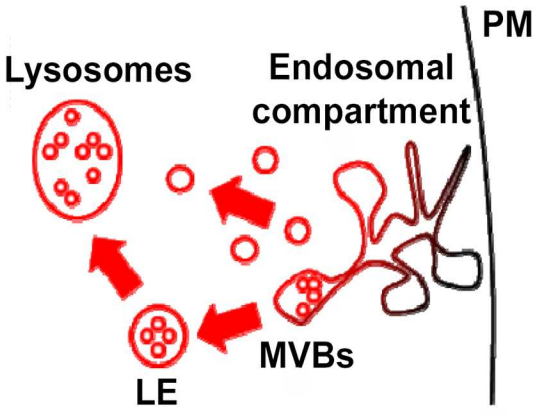

Figure 16: Schematic representation of the PM, the endosomal compartment, the MVBs, the late endosomes (LE) and the lysosomal compartment. The structures involved in endosomal compartment to lysosomes trafficking are represented in red.

Hrs is a monomeric adaptin that has been directly implicated in the biogenesis of the MVBs (Bache *et al.*, 2003). Hrs has affinity to ubiquitinated cargo proteins as well as for the clathrin coat protein (Raiborg *et al.*, 2001). However, unlike other

adaptins, the ability of Hrs to recruit a coat protein does not result in the formation of transport vesicles. Thus, the role of the recruitment of the clathrin coat protein by Hrs in the biogenesis of MVBs is not clear yet. However, Hrs has been found to be part of the ESCRT-0 complex which is a protein complex playing a central role in the biogenesis of MVBs. There are four ESCRT complexes and a description of their roles in the biogenesis of the MVBs is given later in the chapter “The ubiquitin mediated lysosomal down-regulation of integral membrane proteins and the biogenesis of multi-vesicular bodies” of the part VI “The ubiquitination of proteins”. Briefly, the ESCRT complexes are protein complexes which cooperate to sort ubiquitinated cargo proteins into the internal vesicles of the MVBs for subsequent lysosomal degradation (Hurley, 2008). Consequently, ubiquitination at the level of the endosomal compartment is considered to be a signal for transport to the lysosomal compartment (Katzmann *et al.*, 2002). Some proteins can localize in MVBs in an ubiquitination independent manner, but they are usually not incorporated in the internal vesicles and remain on the external membrane of MVBs (Katzmann *et al.*, 2001). Once filled with internal vesicles loaded with cargo proteins, the MVBs are believed to detach from the main endosomal compartment in a annexin II-actin dependent manner (Morel *et al.*, 2009), and to subsequently migrate throughout the cell to ultimately fuse with lysosomes (Luzio *et al.*, 2000). After their detachment from the main endosomal compartment and before their fusion with lysosomes, MVBs undergo a maturation process leading to their acidification and their specific enrichment with lysobiophosphatidic acid (LBPA) (Kobayashi *et al.*, 1998b). Acidic MVBs enriched in LBPA are late endosomes.

As we saw, some membrane proteins rely on ubiquitination for their targeting to the lysosomes, but this is not the case for all membrane proteins. The adaptin AP-3 has been implicated in the transport of some proteins to the lysosomes (Cowles *et al.*,

1997). Similar to the AP-1, AP-2 and AP-4 complex, the AP-3 complex recognizes cargo proteins having YXXθ and [DE]XXXL[LI] protein motifs. However, the properties of the amino acids surrounding the tyrosine residue of the YXXθ protein motif seem to favor a specific interaction with AP-3 rather than with other APs (Ohno *et al.*, 1998). Interestingly, AP-3 can be recruited on both TGN and endosomal membranes (Dell'Angelica *et al.*, 1997) by the small GTPases Arf1 (Ooi *et al.*, 1998), and maybe also Arf5 (Drake *et al.*, 2000). Thus, AP-3 is believed to capture cargo proteins at the level of both the TGN and the endosomal compartment. Although AP-3 is able to interact with the coat protein clathrin (Dell'Angelica *et al.*, 1998), and has been detected on clathrin-coated transport vesicles (Borner *et al.*, 2006; Chapuy *et al.*, 2008), several experimental data demonstrated that AP-3 dependent trafficking does not always require the coat protein clathrin (Shi *et al.*, 1998; Vowels and Payne, 1998). Thus, it is possible that the adaptin AP-3 sustains both a clathrin independent and a clathrin dependent transport route.

The trafficking pathway from the endosomal compartment to the TGN

Only few proteins are known to be transported from the endosomes to the TGN. Consequently, the knowledge about endosomes to TGN protein trafficking consists of a small number of “case report”. There is evidence that two different machineries sustain the transport of biomolecules from the endosomes to the TGN. The SNX proteins-based machinery and the AP-1/clathrin-based machinery. While the AP-1/clathrin machinery is believed to generate clathrin-coated transport vesicles, the SNX proteins-based machinery is rather suspected to generate large tubular-shaped transport carriers (Bonifacino and Hurley, 2008).

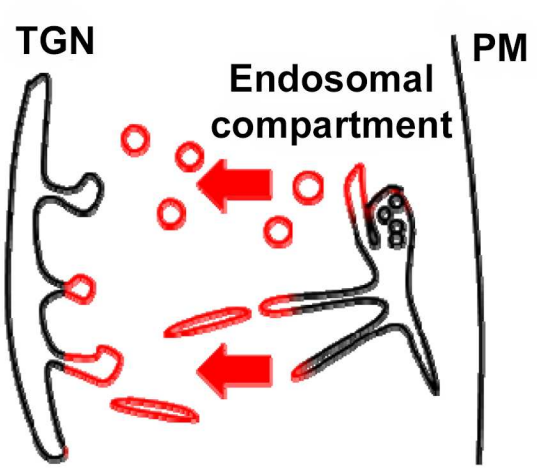

Figure 17: Schematic representation of the PM, the endosomal compartment and the TGN. The structures involved in endosomal compartment to TGN trafficking are represented in red.

In yeast, Vps5p and Vps17p, the homologues of the human SNX1 and SNX2 respectively, are interacting with VPS26p, VPS35p and VPS29p to form the yeast retromer which mediates the retrograde transport of the acid hydrolase receptor VPS10p and the SNARE Yif1p as well as the two peptidases Kex2p and DPAP A (Collins, 2008). Similarly, the human retromer seems to be required for the transport of the CI-MPR from the endosomes to the TGN (Arighi *et al.*, 2004). Other SNX proteins have been implicated in the transport of biomolecules from the endosomes to the TGN. For example, SNX3 is required for the transport of the t-SNARE Pep12 (Hettema *et al.*, 2003) and the amino-peptidase Ste13 from the endosomes to the TGN. Snx4, Snx41 and Snx42 are similarly required for the transport of the v-SNARE Snc1 from the endosomes to the TGN (Voos and Stevens, 1998).

As previously mentioned, there is also evidence that the AP-1/clathrin machinery is implicated in the transport of molecules from the endosomes to the TGN. Mice cells depleted of AP-1 display an impaired transport of the mannose 6-phosphate receptors from the endosomes to the TGN (Meyer *et al.*, 2000). In addition, the trafficking of the chitin synthase Chs3 and the t-SNARE Tlg1 is similarly affected in yeast under the same circumstances (Valdivia *et al.*, 2002).

As a conclusion, two different mechanisms (i.e. SNX-based and AP-1/clathrin-based) are believed to sustain the transport of biomolecules from the endosomes to the TGN.

Part IV: The cation-dependent mannose 6-phosphate receptor

Foreword

The cation-dependent mannose 6-phosphate receptor (CD-MPR) is one of the two mannose 6-phosphate receptors responsible for the sorting of newly synthesized lysosomal enzymes modified with mannose 6-phosphate residues from the TGN to the lysosomes via the endosomal compartment.

The first clue that the sorting of some lysosomal enzymes depends on carbohydrate residues came from the observation that β-hexosaminidase treated with a mild oxidant to oxidize its glycans (e.g. meta-periodate) could not be internalized by human fibroblasts anymore (Hickman *et al.*, 1974). Later on, attempts to inhibit the uptake of β-glucuronidase by human fibroblasts with various sugars revealed that this internalization event could be specifically abolished by the presence of phosphohexosyl residues within the extracellular milieu (Kaplan *et al.*, 1977). Thus, the idea emerged that a phosphohexosyl binding protein could be responsible for this specific internalization event. Shortly after, the α-*N*-acetylglucosaminidase (Varki and Kornfeld, 1980) and the N-acetylglucosaminyl phosphotransferase (Reitman and Kornfeld, 1981) have been found to catalyze the attachment of mannose 6-phosphate residues onto lysosomal enzymes. It then became a priority to identify a membrane protein with a specific affinity to phosphohexosyl residues. This search led to the identification of two discrete membrane proteins that have high affinity for mannose 6-phosphate residues: a two hundred and fifteen kilo Dalton protein called the cation-independent mannose 6-phosphate receptor (CI-MPR) (Sahagian *et al.*, 1981) and a smaller forty-six kilo Dalton protein which is the CD-MPR (Hoflack and Kornfeld, 1985). Since then, a broad range of lysosomal enzymes were found to be decorated with mannose 6-phosphate residues on their

N-glycan structures, and to rely on mannose 6-phosphate receptors for their proper transport throughout the cell (Pohlmann *et al.*, 1995).

Function

Mice lacking the CD-MPR are viable but display a higher level of lysosomal enzymes present in body fluids (Qian *et al.*, 2008). Additionally, fibroblasts isolated from those mice exhibit dense cytoplasmic granules and a swollen endosomal compartment filled with undigested material (Ludwig *et al.*, 1993). All those observations led to the conclusion that the CD-MPR diverts newly synthesized lysosomal proteins from the secretion pathway to reroute them towards the endocytic pathway. To fulfill this function, the CD-MPR binds its cargo proteins at the level of the TGN and use the AP-1/GGA/clathrin machinery to be transported to the endosomal compartment. After releasing its cargo proteins in the endosomal compartment, the CD-MPR recycles back to the TGN for another round of sorting.

The CD-MPR is believed to sort all proteins decorated with mannose 6-phophate residues, and up to two hundred proteins are suspected to bear this particular sugar modification (Sleat *et al.*, 2008). Similarly to the CD-MPR, the CI-MPR also sorts proteins decorated with mannose 6-phophate residues to the lysosomes. The cargo protein spectra of the two MPRs are largely overlapping but some proteins modified with mannose 6-phosphate residues seem to preferentially bind to one of the two receptors (Qian *et al.*, 2008). The main difference between the CD-MPR and the CI-MPR is that the large receptor binds phosphohexosyl residues independently of divalent cations *in vitro*, whereas the binding of phosphohexosyl residues to the CD-MPR *in vitro* is enhanced by divalent cations (Hoflack and Kornfeld, 1985). It is on the basis of this difference that the two mannose 6-phosphate receptors are named cation-independent mannose 6-phosphate receptor and cation-dependent mannose

6-phosphate receptor. However, later work convincingly showed that the CD-MPR can still bind phosphohexosyl residues with high affinity even in the absence of divalent cations (Baba *et al.*, 1988; Junghans *et al.*, 1988).

Altogether, the CD-MPR is a crucial player for the biogenesis and the maintenance of the lysosomal compartment.

Structure

The CD-MPR is a type I transmembrane protein of forty-six kilo Dalton. A leader peptide composed of twenty-eight amino acids drives the translocation of the nascent CD-MPR polypeptide chain into the lumen of the ER where it is subsequently cleaved. The mature CD-MPR comprises three functional domains: an extracellular domain composed of hundred fifty nine amino acids that can bind phosphohexosyl residues, a transmembrane domain composed of twenty five amino acids and an cytoplasmic tail composed of sixty seven amino acids that contains motifs required for its intracellular transport (Figure 18) (Dahms *et al.*, 1987).

Bovine CD-MPR:
MMSPLHSSWRTGLLLLLLFSVAVRESWQTEEKTCDLVGEKGKESEKEL
ALLKRLTPLFNKSFESTVGQSPDMYSYVFRVCREAGNHSSGAGLVQIN
KSNGKETVVGRFNETQIFNGSNWIMLIYKGGDEYDNHCGREQRRAVVM
ISCNRHTLADNFNPVSEERGKVQDCFYLFEMDSSLACSPEISHLSVGS
ILLVTLASLVAVYIIGGFLYQRLVVGAKGMEQFPHLAFWQDLGNLVAD
GCDFVCRSKPRNVPAAYRGVGDDQLGEESEERDDHLLPM

Figure 18: Primary sequence of the bovine CD-MPR. The amino acids of the cleavable leader peptide are indicated in grey, the ones of the luminal domain are in red, the ones of the transmembrane domain are in black and the ones of the cytosolic tail are in blue. The N-glycosylation sites are underlined.

A structural study revealed that the extracellular domain of the CD-MPR is composed of nine anti-parallel β-strands assembling into two orthogonally arranged β-sheets having extensive hydrophobic interactions with each other (Roberts *et al.*,

1998). This study also showed that two CD-MPRs assemble into a homodimer which is stabilized by hydrophobic interactions between the two monomers (Figure 19A).

An additional crystallographic study of the extracellular domain of the CD-MPR bound to a penta-mannosyl phosphate oligosaccharide shed light on the structural basis underlying the recognition of phosphorylated high mannose oligosaccharides by the mannose 6-phosphate binding pocket of the CD-MPR (Figure 19B) (Olson *et al.*, 1999). One of the key features of this binding pocket is its ability to release its ligand once the CD-MPR reaches the endosomal compartment. It has been proposed that this dissociation event would be promoted by the acidic environment of the endosomal compartment. However, crystallographic studies failed to reveal any significant influences of low pH on the mannose 6-phosphate binding pocket of the CD-MPR (Olson *et al.*, 2008). Thus, more investigations are required to understand the mechanisms leading to the dissociation of the CD-MPR and its ligands.

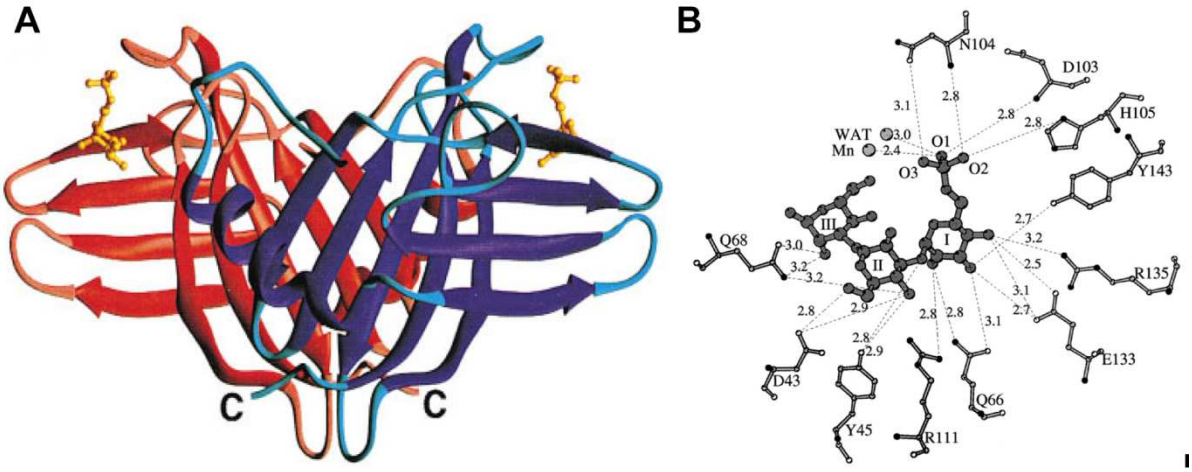

Figure 19: (A) Ribbon diagram of the bovine CD-MPR homodimer. There are two CD-MPR monomers (red/rose ribbon and blue/cyan ribbon) within the asymmetric unit. These monomers are related by a non-crystallographic 2-fold axis parallel to the YZ plane (45° off the x axis). The ligand, Man-6-P, is also shown (gold ball-and-stick model). Adapted from (Roberts *et al.*, 1998). (B) Schematic representation of the potential interactions between penta-mannosyl phosphate oligosaccharide (*dark gray*) and the residues in the binding pocket of the CD-MPR. Potential hydrogen bond distances in angstroms are shown. The terminal (*I*), penultimate (*II*), and prepenultimate (*III*) mannose residues are indicated. Picture from (Olson *et al.*, 1999)

Intracellular transport

At steady state, the CD-MPR population is mainly found in the TGN and in the endosomal compartment (Klumperman *et al.*, 1993). In addition, a minor fraction of the CD-MPR population is located at the plasma membrane (~ 10%). The CD-MPR at the plasma membrane cannot mediate the internalization of ligands for an unknown reason which is independent from the low concentration of divalent cations in the extracellular medium (Stein *et al.*, 1987). Therefore, the biological role of the CD-MPR at the cell surface is not known yet. When it is not at the plasma membrane, the CD-MPR cycles between the TGN and the endosomes to sort newly synthesized lysosomal enzymes (Duncan and Kornfeld, 1988). The transport of the CD-MPR throughout the cell is believed to be mainly sustained by the interaction of the cytoplasmic tail of the receptor with the trafficking machinery of the cell.

The transport of the CD-MPR from the TGN to the endosomes. The results of several work convincingly showed that the CD-MPR interacts with the AP-1/GGA1/clathrin machinery for its transport from the TGN to the endosomes. First, i*n vitro* binding assays and yeast two hybrid studies showed that the adaptins AP-1, AP-3 and GGA1 are able to bind the cytoplasmic tail of the CD-MPR (Honing *et al.*, 1997; Puertollano *et al.*, 2001; Storch and Braulke, 2001; Stockli *et al.*, 2004). In addition, the CD-MPR co-localizes with AP-1 and GGA1 in clathrin-coated structures at the level of the TGN (Klumperman *et al.*, 1993; Puertollano *et al.*, 2001). Finally, a DXXLL protein motif in the cytosolic tail of the CD-MPR can bind the adaptin GGA1 and is necessary for the transport of the CD-MPR from the TGN to the endosomal compartment (Johnson and Kornfeld, 1992). Thus, the idea that the CD-MPR uses the AP-1/GGA1/clathrin machinery to traffic from the TGN to the endosomal compartment is unanimously accepted.

Although AP-3 and CD-MPR can interact *in vitro,* the disruption of AP-3 expression in mouse endothelial fibroblasts does not alter the formation of transport vesicles containing the CD-MPR (Chapuy *et al.*, 2008). Therefore, it is unlikely that AP-3 plays a key role in the vesicular trafficking of the CD-MPR despite the observation that AP-3 and the CD-MPR can interact *in vitro*.

The transport of the CD-MPR from the endosomes to the TGN. Surprisingly, there is also evidence that the CD-MPR relies on the adaptor proteins AP-1 and maybe GGA1 to traffic from the endosomal compartment to the TGN. First, CD-MPRs in fibroblasts of mice deficient for the subunit of the AP-1 complex mu1A exhibit a more peripheral intracellular localization compare to CD-MPRs in wild-type mice fibroblasts (Meyer *et al.*, 2000). In good agreement with this observation, Medigeshi and colleagues (2003) showed that membranes from mu1A-deficient mice fibroblasts fail to sustain the trafficking step of the CD-MPR from the endosomes to the TGN *in vitro*. Finally, a mutant form of the CD-MPR impaired in its ability to bind GGA1 displays a more peripheral intracellular localization in NRK cells compared to the wild-type form of the receptor (Tikkanen *et al.*, 2000). In addition, this mutant form does not efficiently recycle from the endosomes to the TGN. Consequently, the adaptin AP-1 and GGA1 seem to sustain the transport of the CD-MPR between the TGN and the endosomes in both directions.

The di-aromatic protein motif Phe^{18}-Trp^{19} at the position eighteen of the cytosolic tail of the CD-MPR have been also implicated in the endosomal sorting of the receptor (Schweizer *et al.*, 1997). It was proposed that this motif interacts with TIP47 and promotes the transport of the CD-MPR from the late endosomes to the TGN (Diaz and Pfeffer, 1998; Nair *et al.*, 2003). However, this hypothesis has been challenged by an independent study which shows that the efficient transport of the CD-MPR

from endosomes to the TGN is independent from TIP47 *in vitro* (Medigeshi and Schu, 2003). Thus, the implication of TIP47 in the transport of the CD-MPR from endosomes to the TGN is controversial and debated.

Finally, the palmitoylated cystein residue at the position thirty-four (Cys^{34}) of the cytoplasmic tail of the CD-MPR was shown to be required for the efficient transport of the receptor from the endosomal compartment to the TGN. The mutation of this residue to an alanine residue leads to the missorting of the receptor at the endosomal level and its accumulation within dense lysosomes (Schweizer *et al.*, 1996). However, similarly the di-aromatic protein motif Phe^{18}-Trp^{19}, it is not know how the Cys^{34} sustains the intracellular transport of the CD-MPR.

Internalization of the CD-MPR at the plasma membrane. At the plasma membrane the CD-MPR can recruit the adaptin AP-2 (Honing *et al.*, 1997; Storch and Braulke, 2001). Furthermore, the mutation of AP-2 binding sites on the intracellular tail of the CD-MPR leads to its aberrant accumulation at the plasma membrane (Johnson *et al.*, 1990; Schweizer *et al.*, 1997). Therefore, it is unanimously accepted that the CD-MPR is transported from the plasma membrane to the endosomes in clathrin-coated transport vesicles generated by the AP-2/clathrin machinery.

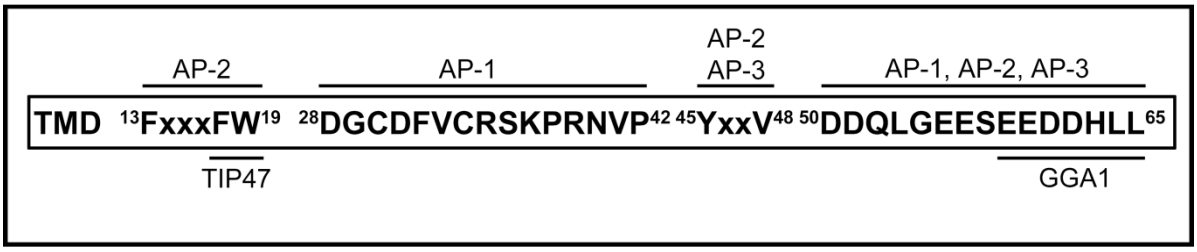

Figure 20: Schematic representation of the cytosolic tail of the CD-MPR. Selected amino acids are indicated in single letter code. The proteins known to interact with the tail of the CD-MPR are indicated over and under the schematic tail and black bars specify with which amino acid of the cytosolic tail of the CD-MPR they are interacting with. TMD: trans-membrane domain

Post-translational modifications

As previously mentioned, the CD-MPR is decorated on its extracellular domain with four N-glycans which have been shown to be heterogeneously modified, resulting in multiple glycosylation forms of the receptor. Of the four N-glycans, usually two to three are of high mannose or hybrid type and one or two are of complex type. Poly-lactosamine sequences and terminal sialic acids residues have been found on certain of those N-glycans (Li and Jourdian, 1991). Although Li and Jourdian (1991) showed that two glycosylation isoforms of the CD-MPR isolated from bovine testis slightly differ in their affinity for mannose 6-phosphate residues, the N-glycans of the CD-MPR do not seem to have a major role in the ability of the receptor to bind phosphorylated mannose residues since a mono-glycosylated truncated form of the receptor displays a affinity for β-glucuronidase similar to the one of the native form of the receptor (Marron-Terada *et al.*, 1998). Thus, the present consensus around the glycosylation of the CD-MPR is that it has no significant role for the stability of the receptor or for its intracellular distribution, but only assists the folding of the CD-MPR into a functional conformation within the ER (Zhang and Dahms, 1993).

The CD-MPR can also be modified with a phosphate residue. It was shown that the serine residue at the position fifty-seven of the cytosolic tail of the CD-MPR is phosphorylated *in vivo* (Hemer *et al.*, 1993). In addition, the casein kinase II has been shown to phosphorylate the CD-MPR *in vitro*, whereas the protein phosphatase 2A can de-phosphorylated it (Korner *et al.*, 1994). Later on, a *in vivo* study showed that a phosphorylation-deficient mutant form of the CD-MPR displays an reduced plasma membrane localization compared to the wild-type form of the receptor (Breuer *et al.*, 1997). Therefore, it was proposed that the phosphorylation of the cytosolic tail of the CD-MPR could modulate its interactions with the vesicular trafficking machinery, thus resulting in a modification of its intracellular localization.

However, an independent *in vitro* study challenged this hypothesis by showing that the phosphorylation of the CD-MPR does not affect its affinity for the adaptins AP-1 and GGA1 (Stockli *et al.*, 2004). Thus, further studies are required to fully understand the role of the phosphorylation of the CD-MPR.

In addition to being phosphorylated, the CD-MPR is also S-palmitoylated, potentially on two cystein residues lying at the position thirty and thirty-four of its cytosolic tail (Schweizer *et al.*, 1996). In the same study, the authors demonstrated that the mutation of the cystein residue at the position thirty-four of the cytoplasmic tail of the receptor leads to the misrouting of the CD-MPR to dense lamp-1 positive membranes, and to the loss of the ability of the receptor to sort pro-cathepsin D. Those results suggest that palmitoylation plays a critical role in the trafficking of the CD-MPR within endosomes. Another work supports this hypothesis by showing that the endosomes and the plasma membrane in HeLa cells contain a palmitoyl transferase activity toward the CD-MPR (Stockli and Rohrer, 2004). It is then proposed that the palmitoylation of the CD-MPR is required for the retrieving of the CD-MPR from the endosomal compartment to the TGN. The mechanism by which the palmitoylation of the CD-MPR promotes this trafficking step is not known.

Part V: Lipid modification of proteins

Foreword

Lipid bilayers have a crucial role in cell biology by forming the plasma membrane and compartmentalizing the cytoplasm. Membrane proteins sustain various crucial biological functions such as the formation of pores within membranes (e.g. members of the aquaporin family) (Khalili-Araghi *et al.*, 2009), the transport of macromolecules throughout membranes (e.g. members of the soluble carrier family) (Kanai and Hediger, 2004) and the transmission of specific signals across membranes (e.g. members of the receptor tyrosine kinase family) (Yarden and Ullrich, 1988). In order to fulfill all those functions, membrane proteins must be able to interact with membranes, sometimes in a reversible manner. The covalent attachment of a lipid to a protein is one of the mechanisms by which the affinity of a protein for a hydrophobic lipid bilayer can be modulated. A protein lipidation can be done on hydroxyl, amine or thiol groups. In this part, the different lipid modifications of proteins are briefly discussed and then emphasis is given to S-palmitoylation which is crucial for this work.

Protein lipidation is a common post-translational modification. The main lipid modifications are acylations, isoprenylations and the addition of glycosylphosphatidylinositol (GPI) anchors.

GPI anchors

Although GPIs are not lipids but glyco-lipids (Figure 21) (Paulick and Bertozzi, 2008), the attachment of a GPI to a protein can be considered as a lipidation due to its functional similarity to acylations and isoprenylations. After being synthesized on ER membranes, the GPI anchors are covalently bound to the C-terminus of a protein bearing a specific carboxyl peptidic signal. The computational analysis of

over hundred-fifty GPI-anchored proteins concluded that the carboxyl signal is a peptide sequence consisting of four sequence regions: a string of roughly ten polar amino acid residues, a cleavage site where the GPI will be covalently attached, a moderately polar string of four to five amino acid residues and a final highly hydrophobic membrane embedded tail (Eisenhaber *et al.*, 1998). The peptidic signal chain is cleaved and the GPI anchor covalently bound to the cleavage site. Altogether, over twenty proteins are required for the synthesis of GPIs and their addition onto proteins (Ikezawa, 2002).

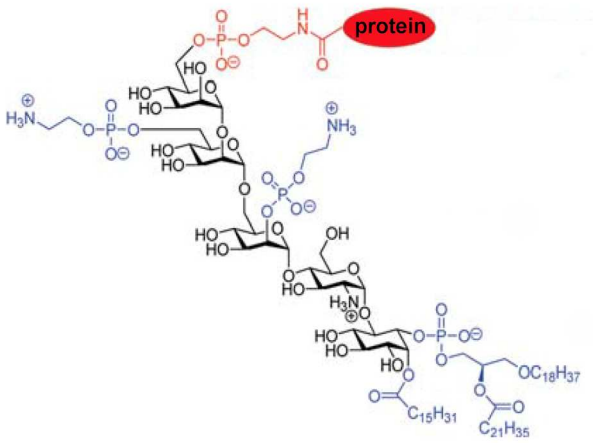

Figure 21: Structure of the GPI anchor. The three domains of the GPI anchor are (I) a phosphoethanolamine linker (red), (II) the conserved glycan core (black), and (III) a phospholipid tail (blue). Appendages in blue (including the lipids of the lipid tail) are variable. Picture from (Paulick and Bertozzi, 2008)

A GPI anchor allows a protein without a transmembrane domain to associate with lipid bilayers and preferentially with detergent resistant sub-domains (Sharom and Lehto, 2002). GPI anchors are considered as stable post-translational modifications although they can be cleaved off by the enzymatic activity of two phospholipases (PLC and PLD) (Huizinga *et al.*, 1988; Hoener *et al.*, 1990), leading to the release of the formerly modified protein from the lipid bilayer.

The isoprenylation of proteins

The isoprenylation of a protein is the attachment of a farsenyl or geranylgeranyl group to the thiol function of a cystein residue (Figure 22) (Clarke, 1992). Three enzymes are known to catalyze protein isoprenylations: farnesyltransferase (FTase), geranylgeranyltransferase I (GGTase I) and geranylgeranyltransferase II (GGTase II) (Fu and Casey, 1999). The two first enzymes catalyze the

isoprenylation of various proteins exhibiting a C-terminal -CaaX protein motif where “C” is a cystein residue and “a” an aliphatic residue. The “X” residue determines whether the motif is recognized by FTase or GGTase I (methionine, glutamine and serine for FTase and leucine for GGTase I). The mono-isoprenylation catalyzed by FTase and GGTase I provides only a modest affinity for lipid bilayers, and an additional membrane binding factor (e.g. additional lipidation, basic amino acid stretch) is usually required to induce an effective membrane association of the modified protein. In contrast, GGTase II transfers a geranylgeranyl moiety to the both cystein residues of -CC or -CxC C-terminal protein motifs present on some proteins, where “C” is a cystein residue and “x” is any amino acid residue. This di-isoprenylation is sufficient for the modified proteins to efficiently bind to lipid bilayers. The activity of GGTase II is believed to be controlled by a chaperone protein Rep which selects and presents substrate proteins to the GGTase II for isoprenylation (Zhang and Casey, 1996).

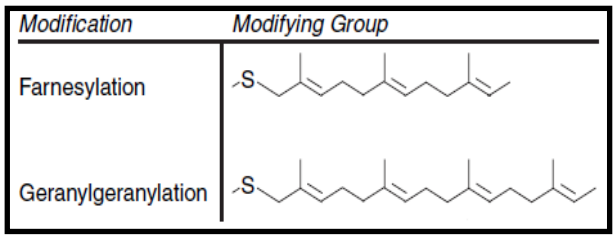

Figure 22: Schematic representation of two isoprenylation: farnesylation and geranylgeralylation

The acylation of proteins

The acylation of a protein is the covalent attachment of a fatty acid (usually myristate or palmitate) to an amine, a thiol or a hydroxyl group of a protein (Figure 23). The most widely spread types of acylation are the N-myristoylation (attachment of a 14-carbon saturated fatty acid to the amine function of the N-terminal glycine of a protein) and the S-palmitoylation (attachment of a 16-carbon saturated fatty acid to the thiol group of a cystein residue). The attachment of a palmitate moiety to the hydroxyl group of a serine residue (Takada *et al.*, 2006) or to the C-terminal amine

group of a protein (Pepinsky *et al.*, 1998; Singh *et al.*, 2004) has also been reported but is believed to be much less frequent than N-myristoylation or S-palmitoylation.

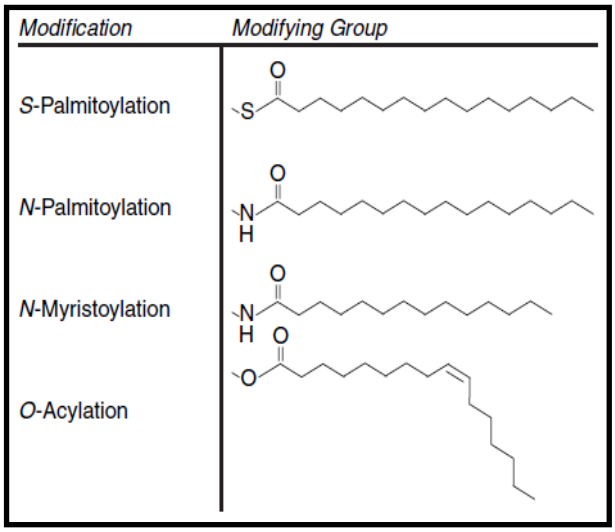

Figure 23: Schematic representation of 4 acylations: S-palmitoylation, N-palmitoylation, N-myristoylation and O-acylation

N-myristoylation is an irreversible co-translational protein modification. However, it can also be post-translational when a protease activity causes the exposure of a new N-terminal of a protein which contains a terminal glycine residue (Zha *et al.*, 2000). Similarly to mono-prenylation, N-myristoylation only promotes a weak and reversible protein-membrane interaction, and additional mechanisms are required to efficiently induce the association of a protein with lipid bilayers (McLaughlin and Aderem, 1995; Resh, 2004). In mammalian species, two N-myristoyltransferases (NMT) have been identified (Type I and Type II) (Farazi *et al.*, 2001), and they enzymatically active on proteins having a N-terminal glycine residue (Maurer-Stroh *et al.*, 2002). The two NMT are highly similar and only diverge at their N-termini which are believed to be responsible for the cellular localization of the NMTs (Glover *et al.*, 1997).

S-palmitoylation, a reversible lipid modification

S-palmitoylation is the covalent thio-ester linkage of a palmitate moiety to the cystein residue of a protein. S-palmitoylation differs from other lipidations by two important aspects. While other lipidations are mostly restricted to peripheral membrane proteins, S-palmitoylation modifies peripheral proteins as well as integral membrane proteins. In addition, S-palmitoylation is readily reversible through the

action of thio-esterases, while other lipid modifications are irreversible modifications although sometime cleavable like GPI-anchors.

Functionally, S-palmitoylation promotes the membrane association of cytosolic proteins and the relocalization of membrane proteins into cholesterol rich membrane sub-domains (Bijlmakers and Marsh, 2003). Therefore, S-palmitoylation directly modulates the intracellular localization of a protein. Through its reversibility, S-palmitoylation provides a mechanism that can actively regulate the functionality of a protein like an ON/OFF switch. Consistently with this idea, S-palmitoylation has been shown to regulate the functions of multiple proteins such as Ras GTPases (Dudler and Gelb, 1996; Hancock, 2003), Rho GTPases (Michaelson *et al.*, 2001; Berzat *et al.*, 2005; Chenette *et al.*, 2005), GPCRs (Chini and Parenti, 2009), SNAREs (Salaun *et al.*, 2005; Valdez-Taubas and Pelham, 2005; He and Linder, 2009), Src kinases (Sandilands *et al.*, 2007) and immunological receptors (Horejsi, 2004).

There is evidence that S-palmitoylation is catalyzed by a family of multipass membrane proteins bearing an Asp-His-His-Cys (DHHC) protein motif within a zinc finger domain (ZDHHC proteins) (Lobo *et al.*, 2002; Roth *et al.*, 2006). The ZDHHC protein family count around eight members in unicellular fungi (Putilina *et al.*, 1999) and usually more than twenty members in metazoans.

It is believed that the DHHC protein motif is the catalytic domain of the ZDHHC proteins because mutations within the DHHC protein motif abolish the catalytic activity of the ZDHHC proteins (Swarthout *et al.*, 2005).

Interestingly, alterations in the expression of several ZDHHC proteins are linked with pathological conditions. For example, the expression of the ZDHHC#2 gene is reduced in colorectal cancer (Oyama *et al.*, 2000), and a single nucleotide polymorphism of the ZDHHC#8 gene is associated with a sub-type of schizophrenia

(Demily *et al.*, 2007). It was also found that the ZDHHC#9 gene is up-regulated in some colon carcinoma sub-types (Mansilla *et al.*, 2007), and that the gain of the ZDHHC#11 genetic locus is associated with bladder and lung cancer (Yamamoto *et al.*, 2007; Kang *et al.*, 2008). In addition, there is evidence suggesting that Huntington disease is caused by the failure of the ZDHHC#17 protein to interact with huntington (Yanai *et al.*, 2006).

There is also evidence that several S-palmitoylated proteins do not rely on ZDHHC proteins for their S-palmitoylation. For example, three mitochondrial enzymes (the carbamoyl-phosphate synthetase 1, the methylmalonate semialdehyde dehydrogenase and the glutamate dehydrogenase) are spontaneously modified with a palmitoyl moiety in the presence of physiological concentrations of palmitoyl-CoA (Berthiaume *et al.*, 1994; Corvi *et al.*, 2001).

In addition to this apparent auto-acylation, it is also possible that some proteins other then ZDHHC proteins could be protein acyl transferases. One indication that such proteins might exist is that the yeast subunit of the TRAPP complex Bet3 (Menon *et al.*, 2006) is expressed in its S-palmitoylated form in E.coli cells which lack ZDHHC genes (Kim *et al.*, 2005). Furthermore, the deletion of multiple ZDHHC genes in yeast does not alter the degree of S-palmitoylation of the endogenous Bet3 (Roth *et al.*, 2006).

As a conclusion, S-palmitoylation is a reversible protein modification which regulates the activity of multiple proteins. Although the enzymology of this modification starts to be revealed, more work is required to fully understand it.

Part VI: The ubiquitination of proteins

Foreword

Ubiquitination is yet another post-translational modification that is specifically mentioned in this work due to its relevance of the CD-MPR. Ubiquitination is the covalent attachment of one or more ubiquitin monomer to a protein via an isopeptidic bond. Ubiquitin is an ubiquitously expressed polypeptide of 76 amino acid residues (8.5kD) which displays an extremely high degree of conservation between species. It was originally isolated from cattle thymus and was mistaken for a hormone (Goldstein *et al.*, 1975). Two years later, it was observed that ubiquitin could covalently modify other proteins (Goldknopf and Busch, 1977) and that this modification was required for the ATP-dependent degradation of abnormal proteins (Etlinger and Goldberg, 1977). Since then, the field of the diverse functions of ubiquitination is constantly expanded. It includes to date cell division, growth, signaling, communication, movement and apoptosis (Hochstrasser, 1996; Hershko and Ciechanover, 1998; Johnson, 2002). Most relevant to this work, ubiquitination also regulates the trafficking of some integral membrane proteins by driving their internalization at the plasma membrane and/or directing them into lysosomes for degradation. In this part, the ubiquitin polypeptide, the mechanisms underlying ubiquitination and finally describe the role of ubiquitination in the biogenesis of multi-vesicular bodies will be introduced.

The ubiquitin polypeptide

As mentioned above, ubiquitin is a 76 amino acid long polypeptide which is ubiquitously expressed and highly conserved from lower eukaryotes to metazoans (Figure 24). For example, the yeast ubiquitin polypeptide and its human counterpart have 96% of sequence identity (Figure 25). The ubiquitin polypeptide has no known

function in itself but can be attached through its C-terminal glycine residue to the lysine residue of various proteins. An additional ubiquitin monomer can be attached to a protein-linked ubiquitin, resulting in protein-linked polyubiquitin chains. The seven lysine residues of the ubiquitin monomer can be used to for glycyl-lysine isopeptidic bonds (Figure 25) (Peng *et al.*, 2003). Once attached to a protein, ubiquitin monomer or polyubiquitin chains are recognized by various proteins bearing an ubiquitin interacting motif (UIM) and the modified protein will be differently processed depending on its intracellular localization and the characteristics of the ubiquitin modification. The nature of the linkage between each ubiquitin monomers and the length of the ubiquitin chain will determine the fate of the ubiquitinated protein.

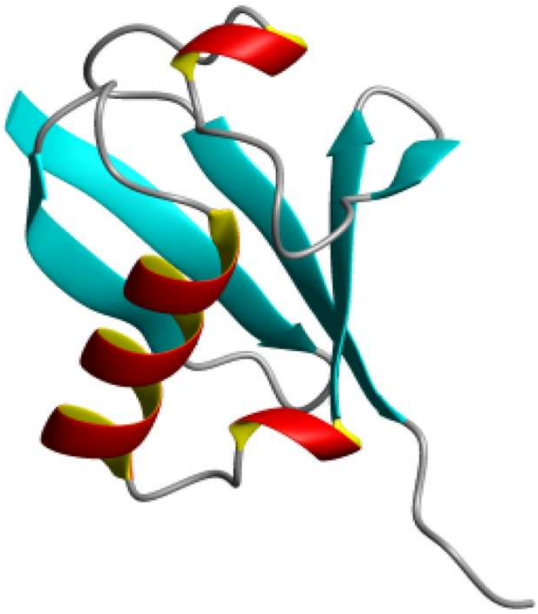

Figure 24: Ribbon diagram of the ubiquitin polypeptide by Vijay-Kumar,S

For example, a polyubiquitin chain in which ubiquitin monomers are linked by Lys48-Gly67 bonds is a signal for proteasomal degradation (Tanaka, 2009).

However, mono-ubiquitination and polyubiquitin chains in which the ubiquitin monomers are linked by Lys63-Gly67 have been implicated in non proteolytic

H.Sapiens	NH_2-MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQ
D.Melanogaster	NH_2-MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQ
S.Cerevisiae	NH_2-MQIFVKTLTGKTITLEVESSDTIDNVKSKIQDKEGIPPDQQ
H.Sapiens	RLIFAGKQLEDGRTLSDYN IQKESTLHLVLRLRGG-COOH
D.Melanogaster	RLIFAGKQLEDGRTLSDYN IQKESTLHLVLRLRGG-COOH
S.cerevisiae	RLIFAGKQLEDGRTLSDYN IQKESTLHLVLRLRGG-COOH

Figure 25: Ubiquitin primary sequence in human, fly and yeast with amino acid residues forming an isopeptidic bond in red.

functions (Sigismund *et al.*, 2004; Chen and Sun, 2009). Finally, the mono-ubiquitination and di-ubiquitination of an integral membrane protein can induce its internalization at the plasma membrane or its delivery to lysosomes for subsequent degradation (d'Azzo *et al.*, 2005).

The ubiquitination machinery

The hallmark of ubiquitination is most likely proteasomal-mediated degradation. By driving the selective down-regulation of poly-ubiquitinated master regulatory cytosolic proteins, ubiquitination plays a major role in important cellular processes such as cell division and apoptosis (Hochstrasser, 1996; Hershko and Ciechanover, 1998). Similarly, ubiquitination also mediates the degradation of receptor tyrosine kinases (RTKs) and G-protein-coupled signaling receptors (GPCRs) by inducing their internalization and trafficking towards lysosomes (Hicke, 1999). In addition, ubiquitin is also involved in the regulation of transcription factors (Kaiser *et al.*, 2000), DNA repair (Hofmann and Pickart, 1999) or ribosomal functions (Spence *et al.*, 2000).

Due to the implication of ubiquitin in such a wide range of important functions, it is crucial for the ubiquitination process in itself to be highly regulated and absolutely precise. This regulation and precision is achieved by the sequential enzymatic action of three proteins: the ubiquitin-activating E1 enzyme, an ubiquitin-conjugating E2 enzymes and an ubiquitin-ligase E3 enzyme (Hershko and Ciechanover, 1998). The ubiquitin-activating E1 enzyme activates an ubiquitin monomer by generating a thiol ester with the carboxyl group of its C-terminal glycine. Once an ubiquitin monomer is activated, it is transferred to one of the several ubiquitin-conjugating enzymes E2 (over thirty in Homo sapiens) which will “carry” it until an ubiquitin-ligase enzyme E3 (hundreds in Homo sapiens) transfers it to a selected target

protein (Figure 26). Subsequently, additional ubiquitin monomers can then be bound to the protein-bound ubiquitin monomer, resulting in a long polyubiquitin chain which is a potent proteasomal-mediated degradation signal (Tanaka, 2009).

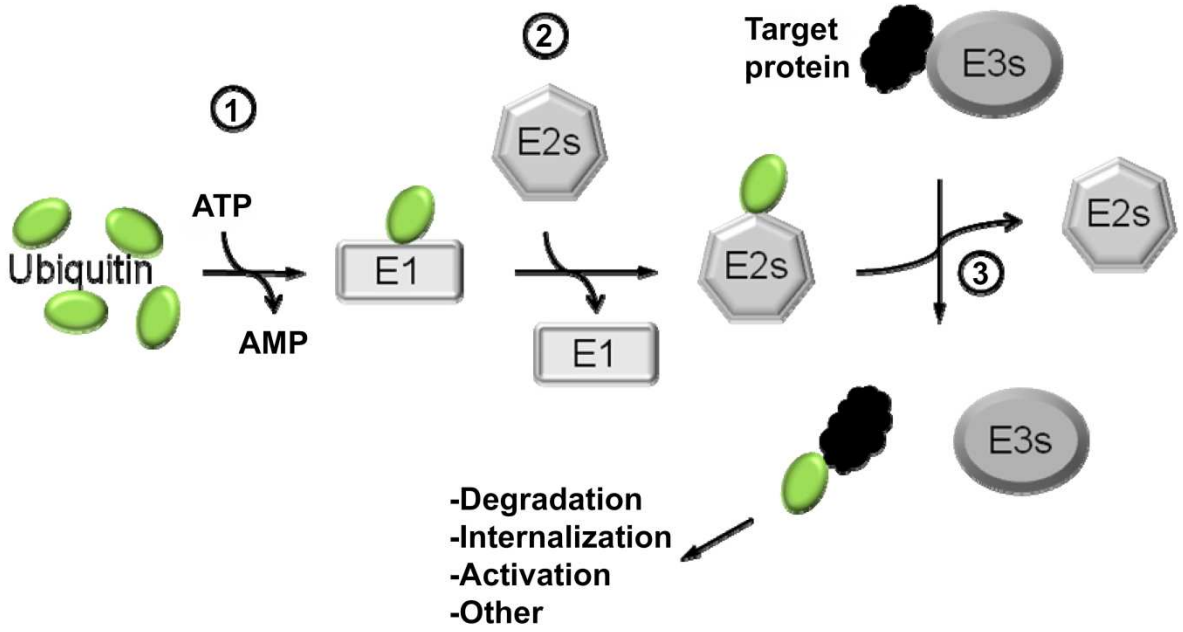

Figure 26: Schematic representation of the sequential enzymatic actions of the ubiquitin machinery. (1) The E1 enzyme activates an ubiquitin monomer and (2) transfers it to an E2 enzyme. The E2 enzyme will "carry" the activated ubiquitin monomer until (3) an E3 enzyme transfers it to a selected target protein.

An additional group of proteins in the enzymology of ubiquitin modifications is the deubiquitinating enzyme (DUB) family. DUBs catalyze the removal and the recycling of ubiquitin monomers from ubiquitinated proteins and also process newly synthesized poly-ubiquitin strings into free ubiquitin monomers (Wiborg *et al.*, 1985). Much like the ubiquitin-ligase E3 enzyme family, the DUB family is extremely diverse with nearly a hundred putative members identified so far (Chung and Baek, 1999), offering an additional layer of regulation to the ubiquitination mechanism. Accordingly, DUBs have been found to be implicated in many ubiquitin functions such as the generation of multi-vesicular bodies, cell growth and differentiation, proteasomal degradation and more (Kim *et al.*, 2003).

Altogether, the ubiquitination mechanism appears to be an extremely well conserved mechanism involved in numerous crucial cellular processes and controlled by very diverse protein families. Most relevant to this work, the ubiquitin system is playing a major role in the lysosomal down-regulation of integral membrane proteins and the biogenesis of multi-vesicular bodies (MVBs).

The ubiquitin mediated lysosomal down-regulation of integral membrane proteins and the biogenesis of multi-vesicular bodies

MVBs form a crucial sub-compartment in the trafficking of ubiquitinated cargo and other proteins towards the lysosomal compartment. Interestingly, the MVBs sub-compartment relies on the ubiquitination machinery for its biogenesis and its maintenance (Bishop and Woodman, 2000; Bache *et al.*, 2003). This link is established by the dual role of an endosomal sorting protein complex required for the transport of ubiquitinated cargo toward the lysosomal compartment and the biogenesis of the MVBs (ESCRT complex) (Katzmann *et al.*, 2001; Bache *et al.*, 2003). The ESCRT machinery includes four protein complexes (ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III) which display a high degree of conservation between species (Leung *et al.*, 2008). ESCRT-0, -I, -II all contain UIMs which allow them to interact with ubiquitinated cargo. In addition to its UIMs, ESCRT-0 also contains a FYVE domain which binds endosomal phosphatidylinositol 3-phosphate (PI3P) (Gaullier *et al.*, 1998) and a clathrin bind motif. Thus, the fact that ESCRT-0 contains a FYVE domain, a clathrin binding motif and IUMs leads to its recruitment onto clathrin-coated endosomal membranes enriched in ubiquitinated cargo (Figure 27A). Once ESCRT-0 is recruited onto the endosomal membrane, ESCRT-I, ESCRT-II and ESCRT-III can then sequentially recruit each other from the cytosol (Figure 27A and B) (Hurley, 2008). The successive actions of the ESCRT

complexes lead to the incorporation of ubiquitinated cargo within vesicles located in the lumen of the endosomal compartment (intra-luminal vesicle (ILV)). It is not known yet how ILVs are formed but the ability of the ESCRT-III protein subunit SNF7 to form filament-like spiral polymers makes it structurally possible that the ESCRT-III participates to the budding of ILV (Figure 27B and C) (Shim *et al.*, 2007). However, the formal proof of such a mechanism is still missing. Just prior to its incorporation within the ILV, the ubiquitinated cargo is de-ubiquitinated and the ubiquitin monomer is recycled by the action of DUBs recruited and activated by the ESCRT-III (Figure 27B) (Agromayor and Martin-Serrano, 2006; Richter *et al.*, 2007). Once the cargo is sorted into the IVLs, the ESCRT-III dissembles and its subunits recycle into the cytosol in a ATP-dependent manner by the action of one more protein complex, VPS4-Vta1 (Figure 27C) (Azmi *et al.*, 2006). The constant incorporation of cargo within ILVs by the ESCRT machinery will ultimately lead to the formation of spherical endosomal sub-compartments filled with ILVs: the MVBs.
The MVBs will ultimately mature into late endosomal compartments and migrate toward the lysosomes in an annexinII/actin dependent manner (Morel *et al.*, 2009) where the cargo incorporated into the ILVs will be degraded by lysosomal proteases.

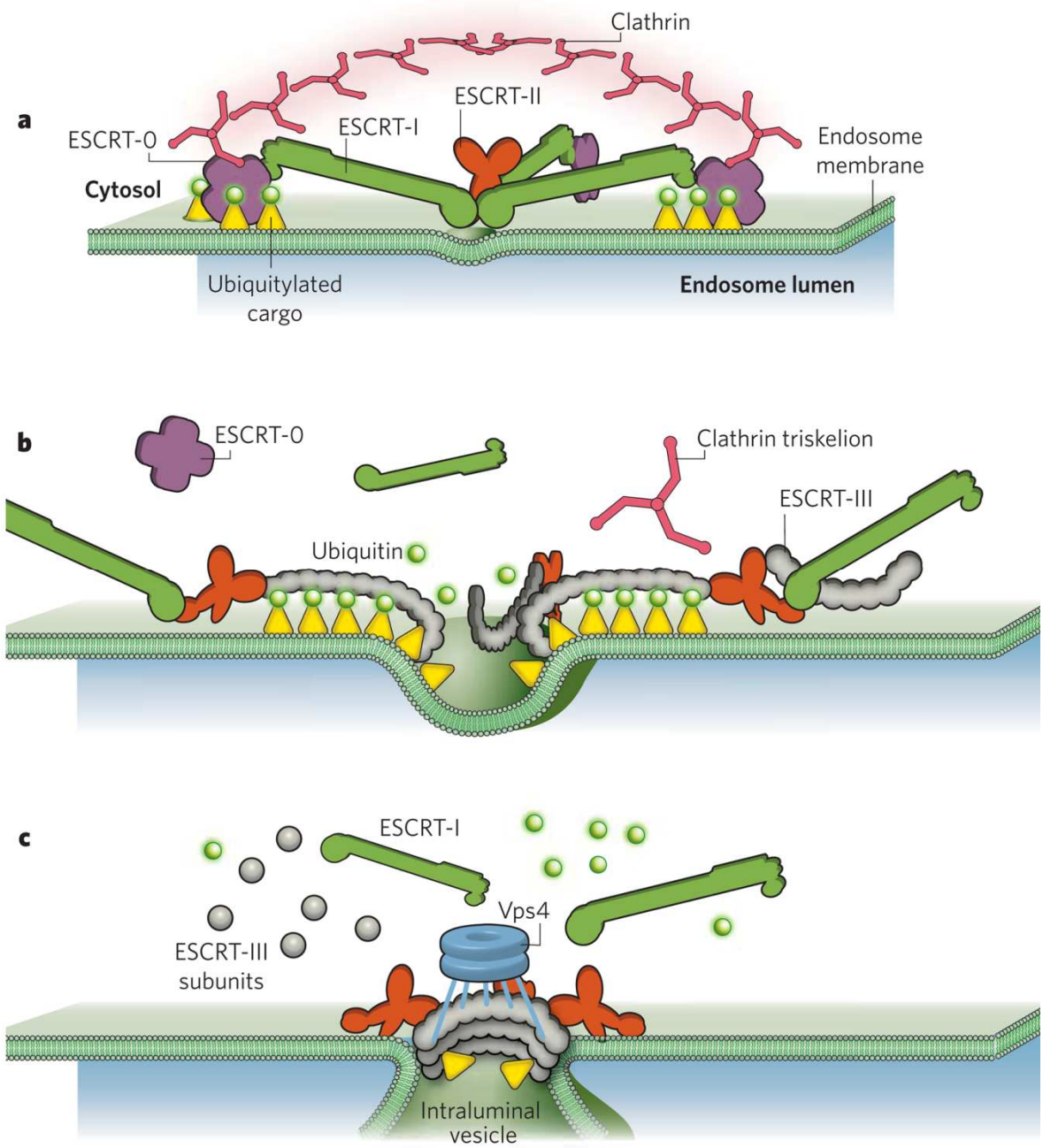

Figure 27: (a) Cargo sorting into clathrin-coated endosomal membranes: initial recognition of ubiquitinated cargo is mediated by ESCRT-0. ESCRT-0 also serves to recruit ESCRT-I. The ESCRT-I recruits ESCRT-II (B) Membrane deformation: ESCRT-III complexes are recruited by binding ESCRT-II and form spiral-shaped filaments which may promote the budding of ILVs. During this process, cargo is de-ubiquitinated by DUBs that are recruited by ESCRT-III and incorporated into the nascent ILV. (c) Membrane scission: as ESCRT-III filaments assemble into circular arrays, the membrane continues to invaginate. Vps4 enters the invagination to disassemble ESCRT-III filaments, ensuring that its subunits are recycled and that the filaments assemble only at the neck of the forming intra-luminal vesicle. For simplicity, the cytosolic part of the transmembrane cargo (yellow) has been omitted in the figure. Picture from (Raiborg and Stenmark, 2009).

Results

Part I

The cation-dependent mannose 6-phosphate receptor is ubiquitinated and palmitoylated for its sorting in endosomes

Bruno G. H. Filippi[*], Alissa C. Vesecky[†], Adriano Marchese[†], Fabrizio Vacca[§], Christian Caprara[*], Marek Whitehead[*], Jean Gruenberg[§] and Jack Rohrer[*‖]

[*]Institute of Physiology, University of Zurich, Zurich, Switzerland.

[†] Department of Pharmacology, Stritch School of Medicine, Loyola University Chicago, Maywood, IL 60153, United State of America.

[§] Department of Biochemistry, University of Geneva, 30 quai Ernest Ansermet-1211 Geneva 4, Switzerland.

[‖] Corresponding author: email: roja@zhaw.ch Present address: Institute for Biotechnology, Zurich University of Applied Sciences, Wadenswil, Switzerland

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Summary

Background: The cation-dependent mannose 6-phosphate receptor constitutively cycles between the trans-Golgi network and the endosomes to deliver newly synthesized mannose 6-phosphate tagged lysosomal enzymes from the trans-Golgi network via endosomes to the lysosomal compartment. Sorting of the cation-dependent mannose 6-phosphate receptor within the endosomal compartment has been shown to require the S-palmitoylation of a cystein residue in its cytoplasmic tail to avoid delivery of the receptor itself to the lysosomes. Methodology/Principal Findings: In this work, we demonstrate that this reversible post-translational modification allows the transient association of the cation-dependent mannose 6-phosphate receptor with detergent resistant membrane subdomains which are not localized at the plasma membrane. Additionally, using a FLAG-tagged ubiquitin approach, we could show that the cation-dependent mannose 6-phosphate receptor can be ubiquitinated. The ubiquitination of the cation-dependent mannose 6-phosphate receptor also occurs at the plasma membrane and is not required for the internalization of the cation-dependent mannose 6-phosphate receptor from the plasma membrane. Conclusions/Significance: Altogether, these results suggest that the retrieval of the cation-dependent mannose 6.phosphate receptor from the endosomal compartment to the trans-Golgi network requires the association of the receptor with detergent resistant membrane sub-domains located within the early endosomes via S-palmitoylation. Additionally, an ubiquitin ligase activity for the cation-dependent mannose 6.phosphate receptor exists at the plasma membrane, suggesting that ubiquitination might be involved quality control mechanisms of non functional receptors leading to lysosomal degradation.

Introduction

The mannose 6-phosphate receptors are type I integral membrane proteins that sort newly synthesized lysosomal enzymes tagged with a mannose 6-phosphate residue from the trans-Golgi network (TGN) to the lysosomes (Dahms and Hancock, 2002). To fulfill their sorting function, the mannose 6-phosphate receptors capture their cargo protein within the TGN, are subsequently transported to the endosomes where their cargo is released, and are finally retrieved to the TGN for an additional round of sorting. Up to now, two different mannose 6-phosphate receptors have been identified: the cation-dependent mannose 6-phosphate receptor and the cation-independent mannose 6-phosphate receptor (CD-MPR and CI-MPR, respectively) (Qian *et al.*, 2008). In this work, we focus on the CD-MPR, which cycles between the TGN and the endosomes by interacting with the AP/GGA/clathrin machinery through small amino acid sorting motifs located in its sixty-seven amino acid residues long cytoplasmic tail (Ghosh *et al.*, 2003). The CD-MPR is ubiquitously expressed, and its deletion leads to an aberrant secretion of lysosomal enzymes (Koster *et al.*, 1993; Ludwig *et al.*, 1994).

After having delivered its cargo in the endosomal compartment, the CD-MPR is quickly recycled from the endosomes to the TGN (Duncan and Kornfeld, 1988). To achieve this transport step, the CD-MPR needs to be efficiently sorted within the endosomes (intra-endosomal sorting). The endosomal compartment is a complex organelle, and the molecular mechanisms sustaining the intra-endosomal sorting of proteins are still poorly understood, despite intense research efforts (Gruenberg, 2001; van der Goot and Gruenberg, 2006). Therefore, the mechanisms regulating the intra-endosomal sorting of the CD-MPR have not been delineated in detail, although this trafficking step has been proven to be important for the stability of the receptor (Rohrer *et al.*, 1995; Schweizer *et al.*, 1997) as well as for its biological

function (Schweizer *et al.*, 1996). A cystein residue located at the position thirty-four of the cytosolic tail of the CD-MPR (Cys^{34}) is implicated in this intra-endosomal trafficking step (Schweizer *et al.*, 1996). Interestingly, this cystein residue has also been found to be S-palmitoylated at the plasma membrane (PM) and in the endosomes (Stockli and Rohrer, 2004). S-palmitoylation is the reversible thioester linkage of a 16-carbon saturated fatty acid to the thiol group of a cystein residue, and plays an important role in the trafficking of many membrane proteins by inducing membrane anchoring and/or association with detergent resistant membrane (DRM) sub-domains (Resh, 2006; Linder and Deschenes, 2007). S-palmitoylation is believed to act through the insertion of the highly hydrophobic palmitate moiety into the lipid bilayer.

In the context of the trafficking of the CD-MPR, it has been suggested that the reversible S-palmitoylation of the Cys^{34} could anchor the center of the cytoplasmic tail of the receptor in the lipid bilayer, and thus modulates the presentation of the sorting motifs of the receptor (Schweizer *et al.*, 1996). Another hypothesis is that S-palmitoylation could increase the hydrophobicity of the CD-MPR, which would target the receptor to specialized DRM sub-domains where the machinery required for its proper trafficking would be located.

An additional reversible post-translational modification which regulates protein trafficking is the covalent attachment of ubiquitin, a conserved 76-amino acid peptide to a lysine residue of a substrate protein. The endosomal trafficking of many membrane proteins (e.g. G protein-coupled receptors, receptor tyrosine kinases) has been shown to depend on the ubiquitin machinery through their mono-, multi- or poly-ubiquitination (Staub and Rotin, 2006; Marchese *et al.*, 2008).

In this work, we analyzed the role of both S-palmitoylation and ubiquitination in the intra-endosomal sorting of the CD-MPR. We show that a small subpopulation of the

CD-MPR is associated with DRMs most probably located within early endosomes and that S-palmitoylation is required for this association. Furthermore, it was found that the CD-MPR is ubiquitinated on lysine residues within its intracellular tail and that this modification can occur at the PM without being required for internalization, whereas de-ubiquitination might occur in early endosomes.

Materials and methods

Materials

Chemicals were from AppliChem (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, U.S.A.). OptiPrep and protein inhibitor cocktail (SigmaPIC) was obtained from Sigma-Aldrich. Nitrocellulose was from Whatman PLC (Florham Park, U.S.A.). Dulbecco's modified Eagle medium (DMEM) and bovine fetal calf serum (FCS) were from Invitrogen (Carlsbad, U.S.A.). Disposable plastic ware and cell culture dishes were from TPP (Trasadingen, Switzerland), VWR (Wien, Austria), Greiner Bio One (Monroe, U.S.A.) or Bioswisstec (Schaffhausen, Switzerland). Low-fat dry milk powder was from Coop (Basel, Switzerland).

Antibodies

The anti-caveolin1 antibody used was the polyclonal antibody N-20 from Santa Cruz (Santa Cruz, U.S.A); the anti-transferrin receptor antibody was the monoclonal antibody 13E4 from Santa Cruz; the anti-LBPA antibody was the monoclonal antibody 6C4 and has been described previously (Kobayashi *et al.*, 1998b); the monoclonal antibody against Rab5 was a gift from R. Jahn (Göttingen, Germany); the anti-bCD-MPR antibody was the monoclonal antibody 22D4 purified from hybridoma supernatant (Messner, 1993), all reducing agents were omitted when using 22D4; the anti-biotin-HRP antibody was a polyclonal antibody from Sigma-Aldrich; the anti-FLAG antibody was the monoclonal antibody M2 from Sigma-

Aldrich; the sheep anti-mouse IgG HRP antibody was “ECL Anti-mouse IgG Horseradish peroxidase linked whole antibody from sheep” from GE healthcare (Chalfont St.Giles, UK).

Common solutions

Unless specified differently, solutions are made in ddH_2O. Phosphate buffer saline (PBS) [3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, pH 7.4]; tris-buffered saline (TBS) [TBS: 50 mM Tris-HCl; 150 mM NaCl, pH 7.4]; tris-buffered saline EDTA (TNE) [150mM NaCl, 2mM EDTA, 50mM Tris–HCl, pH 7.4]; sample buffer (SB) [37.5 mM Tris-HCl, 8% SDS, 10% glycerol, 0.003% bromophenol blue, 50mM DTT, pH 6.5]; 500×PIC (5mg/ml benzamidine, and 1µg/ml of pepstatin A, leupeptin, antipain, and chymostatin in 40% dimethylsulfoxide-60% ethanol)

SDS-PAGE, Western blotting and immuno-detection

SDS-PAGE electrophoresis of samples was executed accordingly to Laemmli (1970). Western blot was performed accordingly to Towbin et al (1979). Unless differently specified, all immuno-detection steps were performed at room temperature. Prior to immuno-detection, the nitro-cellulose membrane resulting from a Western blot was blocked with a solution of three percent milk (w/v) in PBS (MPBS) for one hour and subsequently rinsed three times for thirty seconds with PBS. Immuno-detection of bCD-MPR was performed with the monoclonal antibody 22D4 at a concentration of 1 µg/ml in MPBS for one hour. After the incubation, the nitro-cellulose membrane was washed three times for five minutes in MPBS and the primary antibody was detected with a 1/2000 dilution of a polyclonal sheep antibody to mouse IgG coupled with HRP in MPBS for one hour. Finally, the membrane was washed three times ten minutes in PBS. Immuno-detection of biotin was performed with over-night incubation in a 1/500 dilution of polyclonal antibody α-biotin

crosslinked with HRP in a solution of 3% BSA (w/v) in PBS at 4℃. After the incubation, the nitro-cellulose membrane was washed three times twenty minutes in PBS. Immuno-detection of FLAG epitope was performed with the monoclonal antibody M2 at a concentration of 5µg/ml in a solution of 0.05% TWEEN20 (v/v) in TBS (TBST) for one hour. After the incubation, the nitro-cellulose membrane was washed three times five minutes in TBST and the primary antibody was detected with a 1/10000 dilution of a polyclonal antibody goat α-mouse crosslinked with HRP in a solution of 5% milk (w/v) in TBST for one hour. Finally, the nitrocellulose membrane was washed five times ten minutes in TBST. Unless differently specified, the final detection of HRP-conjugated immuno-globulins adsorbed on the nitro-cellulose membranes was done by using a chemiluminescent substrate (Supersignal West Pico**,** Pierce, Rockford, U.S.A) according to manufacturer's instructions and subsequently exposing the nitro-cellulose membrane to an autoradiography film (Kodak biomax light film, Sigma-Aldrich).

TCA precipitation

The sample was first adjusted to 0.02% (w/v) deoxycholate from a 0.2% (w/v) stock in ddH_20, then adjusted to 15% (w/v) trichloroacetic acid from a 100% (w/v) stock in ddH_20 and vigorously mixed. After incubation for thirty minutes on ice, the sample was centrifuged at 16.1 krcf for fifteen minutes at 4℃ to pellet precipitated proteins. The supernatant was discarded and the pellet washed twice with ice-cold acetone. The pellet was then air dried and resuspended in SB.

Isolation of detergent-resistant membranes

HeLa cells stably expressing a wild-type or a mutant form of the bovine CD-MPR (Wt, MPR $C^{30}C^{34}$-A, MPR $K^{8}K^{37}$-R or MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A) were grown to reach 90% confluence in a 3.5 cm tissue culture well. All the following steps were carried out on ice in a 4℃ room with ice-cold buff ers. Cells were washed once with

PBS and TNE, scraped in 1mL TNE, and subsequently pelleted at 380rcf for five minutes at 4℃. The cell pellet was resuspended in 250 µL of TNE/PIC/ 400 µM PMSF and homogenized by passing twenty-five times through a 25G needle. Hundred ninety eight micro liters of cell lysate were transferred to a new microcentrifuge tube and 22 µL of 10% (w/v) Triton X-100 (Surfact-Amps X-100; Pierce) were added to reach a final Triton X-100 concentration of 1% (w/v). The samples were then mixed by gentle inversion and solubilized on ice for thirty minutes. Subsequently, the samples were adjusted to 40% (w/v) iodixanol by adding 440 µL of a 60% (w/v) iodixanol solution (Optiprep) and 600 µL of the sample were transferred to a centrifugation tube (2.2 mL Ultra-Clear™ Tubes, Beckman-Coulter) followed by an overlay with 1200 µL of a 30% (w/v) iodixanol solution prepared in TNE and finally with 200 µL TNE on the top. The samples were then centrifuged at 220 krcf with a pre-cooled TLS55 rotor for 2.5 hours at 4℃. After centrifugation, two fractions of 1 mL were collected from the top of the gradient. The upper fraction contains the detergent-resistant membranes (DRMs) and the lower fraction contains the detergent-sensitive membranes (DSMs). Each fraction was supplemented with 50 µl of 10% (w/v) Triton X-100 and incubated on an end over end shaker for 15min at room temperature to solubilize the DRMs. Finally, proteins of the two fractions were concentrated by TCA precipitation. To determine precisely the amount of receptor localized in the DRM, a serial dilution of the DSMs fraction was compared with 50% of the DRM fraction. The samples were resolved on a SDS-PAGE, transferred onto a nitro-cellulose membrane by Western blot and caveolin 1, transferrin receptor or bCD-MPR was immuno-detected.

Protein biotinylation assay

HeLa cells stably expressing a wild-type or a mutant form of the bovine CD-MPR (Wt, MPR $C^{30}C^{34}$-A, MPR $K^{8}K^{37}$-R or MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A) were grown to

reach 90% confluence in a 3.5 cm tissue culture well. All the following biotinylation steps were carried out on ice in a 4℃ room with ic e-cold buffers. The cells were washed twice with PBS and proteins were biotinylated by incubating the cells two hours in PBS supplemented with 1 mM sulfo-NHS-biotin in the presence or in the absence of 0.05% saponin, for total and PM protein biotinylation respectively. After the biotinylation step, the cells were washed twice with 1 ml of PBS supplemented with 5 mM glycine (GPBS) and the remaining biotinylation agent neutralized by fifteen minutes incubation in 1 ml of GPBS. Then, the cells were solubilized in the well by adding 1.3 mL of lysis buffer (PBS, 0.5% Triton X-100, 5 mM glycine, 1 mM EDTA, SigmaPIC, pH 7.2) and gentle shaking for ten minutes at 4℃. The lysate was then passed five times through a syringe using a G25 needle and transferred to an Eppendorf tube. After twenty additional minutes of solubilization on ice, the sample was centrifuged at 23 krcf for 15 min at 4℃. The supernatant was collected and the pellet discarded. The sample was incubated for 2 hr at 4℃ with 35 µl of proteinA-22D4 DMP crosslinked beads on an end over end shaker to immuno-precipitate the bCD-MPR. Subsequently, the beads were washed three times ten minutes with 1 ml of PBS / 0.1% Triton X-100. Finally, the samples were resolved on a 10% SDS-PAGE, transferred onto a nitro-cellulose membrane by Western blot and biotin was immuno-detected. Chemiluminescence signals emitted during the immuno-detection of biotin were quantified using a DIANA III chemiluminescence detection system (Raytest, Straubenhardt, Germany)

Ubiquitination Assay

For the detection of ubiquitinated bovine CD-MPR (bCD-MPR), HEK293 cells grown to 60-70% confluence on 10 cm dishes were transiently transfected using either FuGene-6 (Roche, U.S.A.) or TransIT-293 (Mirus, U.S.A.) transfection reagents (according to the manufacturer's instructions) with 0.5 µg of plasmid DNA encoding

the bCD-MPR (Wt, MPR $C_{30}C_{34}$-A, MPR K_8K_{37}-R or the empty vector (pcDNA)) and 3 µg of plasmid DNA encoding a FLAG-tagged ubiquitin (Marchese and Benovic, 2001). Following transfection, cells were grown for an additional forty-eight hours. Subsequently, the plates were placed on ice, the media was aspirated and cells were scraped in 1ml of ice-cold lysis buffer [50mM Tris-HCl (pH8), 150mM NaCl, 1% Triton-X100 (v/v), 20mM NEM, 10µg/ml pepstatin A, 10µg/ml leupeptin, 10µg/ml aprotinin]. Samples were transferred into fresh microcentrifuge tubes and solubilized by rocking for thirty minutes at 4˚C. Subsequently, the samples were sonicated on ice for ten seconds at setting 10% (Branson Digital Sonifier 450) and clarified by centrifugation at 21 krcf for 20 min at 4˚C. An aliquot of the cleared lysate was taken to assess the expression of the various constructs by Western blot and immuno-detection with the monoclonal antibody M2 for FLAG-ubiquitin and with the monoclonal antibody 22D4 for bCD-MPR.

The bCD-MPR was immunoprecipitated from 600 µl of the cleared lysate (rocking incubation with the monoclonal antibody 22D4 at a final concentration of 3 µg/ml for one hour at 4℃, followed by an overnight rocking i ncubation at 4˚C with 20 µl of a 1:1 ratio of proteinG beads). Subsequently, beads are briefly washed twice with 750 µl lysis buffer and bound proteins are eluted at room temperature for 30 min with 20 µl of 2× SB. Finally, samples were resolved on a SDS-PAGE, transferred onto a nitro-cellulose membrane by Western blot and FLAG-epitope was immuno-detected. After the immuno-detection of the FLAG-epitope, the nitro-cellulose membrane was stripped by two 15 minutes rocking incubation at 50℃ in a solution of 0.2M glycine/ 2% (w/v) SDS pH2 and the total bCD-MPR was immuno-detected using the monoclonal antibody 22D4.

Subcellular fractionation and late endosomal membranes isolation

Late endosomal fraction was prepared as described (Aniento *et al.*, 1996) with minor modifications. Briefly, BHK cells were homogenized, a post-nuclear supernatant was prepared and adjusted to 40.6% sucrose, 3 mM imidazole, pH 7.4, loaded at the bottom of an SW60 tube, and overlaid sequentially with 35% and 25% sucrose solutions in 3 mM imidazole, pH 7.4, and then homogenization buffer (HB; 8.5% sucrose, 3 mM imidazole, pH 7.4). The gradient was centrifuged for 60 min at 35.000 rpm. Early and late endosomal fractions were collected at the 35%/25% and 25%/HB interfaces respectively. Quantification of LBPA content of fractions was performed as previously described (Kobayashi *et al.*, 1998b). For Western blot and ELISA analysis equal volumes of early and late endosomal fractions were loaded on gel (corresponding approximately to 5 µg and 15-20 µg of late and early endosomal proteins respectively).

Results

Cystein 30 and 34 are required for localization in detergent resistant membranes

Association of transmembrane proteins with membrane sub-domains upon S-palmitoylation is a known phenomenon; see Zhang et al. (1998) and Fragoso et al (2003) for some pertinent examples. Thus we hypothesize that the S-palmitoylation of the CD-MPR at the endosomal level could target the CD-MPR into specialized endosomal DRMs.

To test this hypothesis, the DRMs of different HeLa cell lines stably transfected with the wild-type or a mutant form of the bovine CD-MPR (bCD-MPR) were isolated. Caveolin 1, a marker for DRMs (Brown, 2006), was localized completely within the DRM fraction which ensures that the DRMs were still intact after the extraction procedure (Fig.2B, upper blot). The transferrin receptor which should be excluded

from the DRMs (Brown, 2006) was used as an additional control to ensure that the detergent soluble membranes were completely solubilized (Fig.2B, lower blot). For a better comparison of the amount of receptor found in the DRM fraction versus the DSM fraction, a serial dilution of the DSM fraction was blotted within a range to have the signal intensity of the DSM fraction matching about the signal intensity of the DRM fraction. We found that a significant fraction (>10%) of the total wild type bCD-MPR population co-extracts with DRMs (Fig.2A, wt lane). To determine the role of S-palmitoylation for this association of the CD-MPR with DRMs, a palmitoylation-deficient bCD-MPR mutant (Fig.1, MPR $C^{30}C^{34}$-A) (Schweizer *et al.*, 1996) was tested for DRM association. Clearly, the palmitoylation-deficient receptor, MPR $C^{30}C^{34}$-A, did not co-extract with DRMs. Less than 5% from total pool of MPR $C^{30}C^{34}$-A was found in the DRM fraction compared to the DSM fraction (Fig.2A, MPR $C^{30}C^{34}$-A lane). Therefore, we conclude that S-palmitoylation is required for the CD-MPR to associate efficiently with DRMs.

Since the CD-MPR is S-palmitoylated at the PM and in the endosomes (Stockli and Rohrer, 2004), we wanted to analyze if the association of the CD-MPR with DRMs occurs mainly at the PM, at the endosomal level or both. To test this, we determined the ability of a bCD-MPR mutant with all potential internalization signals replaced by alanine residues (Fig. 1, MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A) to co-extract with DRMs. This mutant form of the bCD-MPR is showing not only an aberrant localization with more than 85% of the receptor present at the PM compared to about 10% present at the PM for the Wt form of the receptor but it has also a high level of palmitoylation (Stockli and Rohrer, 2004). Therefore, if the CD-MPR is associated with DRMs at the PM, this mutant form of the bCD-MPR should display an even stronger association with DRMs compared to the wild type bCD-MPR. Surprisingly, the MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A mutant shows only a weak association with DRMs (5%-10%

of the total MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A pool) (Fig.2A, MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A lane). This finding supports the view that the association of the CD-MPR with DRMs does not primarily occur at the PM but in the endosomal compartment.

To further analyze the association of the CD-MPR with DRMs at the level of the endosomal compartment, we took advantage of an established protocol which allows to separate early and late endosomal membranes isolated from BHK cells using a sucrose step gradient (Aniento *et al.*, 1996). As shown in Fig. 3A, a transiently expressed wild type bCD-MPR can be found in an early endosomal fraction as shown by the co-purification with Rab5, a small GTPase involved in the organization of the early endocytic pathway (Bucci *et al.*, 1992). The purified late endosomal membrane fraction is highly enriched in LBPA (Fig. 3B), a lipid mainly found in internal membranes of late endosomes (Kobayashi *et al.*, 1998b), and devoid of Rab5 (Fig. 3A, bottom panel). However, we failed to detect a transiently expressed wild type bCD-MPR in the late endosomal membrane fraction of BHK cells (Fig. 3A). Thus, we concluded that it is indeed at the level of early endosomes where the CD-MPR encounters a rate limiting sorting event and most likely associates with DRMs in early endosomes.

CD-MPR is ubiquitinated

Previous work demonstrated that some mutant forms of the CD-MPR with deletions of a short amino acid stretch within their cytoplasmic tail have a reduced half-life compared to the wild type CD-MPR but can be stabilized by a proteasomal inhibitor, lactacystin (Breuer and Braulke, 1998). This sensitivity to lactacystin suggests an involvement of the ubiquitination machinery in the trafficking of the CD-MPR. The intracellular tail of the CD-MPR contains two lysine residues which are potential acceptors of an ubiquitin modification at position 8 and 37 from the transmembrane

domain (Fig.1, Wt). This prompted us to consider that the CD-MPR could be ubiquitinated and that this ubiquitination could play a role in the trafficking of the CD-MPR.

Thus, a FLAG-tagged ubiquitin labeling approach was used to test if the CD-MPR is ubiquitinated. Our results show that, in presence of FLAG-ubiquitin, the wild type bCD-MPR incorporates FLAG epitopes (Fig.4A, lane 1) whereas the mock treated samples do not (Fig.4A, lane 5) and that the majority of the FLAG-tagged wild type bCD-MPR is ~15 kD larger compared to the unmodified wild type bCD-MPR form (Fig.4A, lane 1 versus 4B, lane 1). In addition, this incorporation of FLAG epitopes into the receptor was no longer detected when the only two lysine residues located within the cytoplasmic tail of the bCD-MPR were exchanged for arginine residues (Fig.4A, lane 4). Taken all together, we conclude that the CD-MPR is di-ubiquitinated on its cytoplasmic tail.

Ubiquitination of some transmembrane proteins is known to occur at the PM for internalization and subsequent intra-endosomal sorting (Staub and Rotin, 2006; Acconcia *et al.*, 2009). To test if the ubiquitination of the CD-MPR occurs at the PM, incorporation of FLAG-tagged ubiquitin into the bCD-MPR mutant defective for internalization (Fig.1, MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A) was analyzed. As for the DRM localization, an ubiquitination at the PM should result in a higher level of ubiquitination of this bCD-MPR mutant. Using the same FLAG-tagged ubiquitin approach; we observed that this mutant form of the bCD-MPR displays a very strong ubiquitination compared to the wild type (Fig 4A, lane 3 versus lane 1).

Since the ubiquitination of the CD-MPR can occur at the PM, we considered the possibility that ubiquitination could play an important role for the internalization of the CD-MPR. If this hypothesis is correct, the ubiquitination deficient bCD-MPR mutant (Fig.1, MPR $K^{8}K^{37}$-R) should exhibit a stronger PM localization compared to

the wild type due to its failure to internalize properly. To examine this, HeLa cell lines stably transfected with the wild-type or a mutant form of the bCD-MPR were biotinylated with a membrane impermeable biotinylation agent in the presence or the absence of saponin, a permeabilization agent. In the absence of saponin, only a minority of the wild type bCD-MPR was accessible to the biotinylation agent (Fig.5, lane 4), showing that only about 10% of the wild type bCD-MPR population is located at the PM which is in good agreement with previous work (Schweizer *et al.*, 1997). Conversely, the internalization deficient bCD-MPR mutant displayed a strong PM localization using this approach (Fig 5B, lane 4). However, the ubiquitination deficient bCD-MPR mutant (Fig.1, MPR $K^{8}K^{37}$-R) displayed a localization to the PM similar to the one of the wild type (compare Fig.5A, lane 4 versus Fig 5C lane 4). This result demonstrates that ubiquitination is not required for the efficient internalization of the CD-MPR at the PM.

Discussion

The presented results in this study demonstrate that the CD-MPR associates with DRMs, most likely in early endosomes, and that the palmitoylated cystein residues of the cytoplasmic tail of the receptor (C^{30} and C^{34}) are required for this association. In addition, we found that the CD-MPR is di-ubiquitinated on its intracellular tail, which also occurs at the PM but is not required for internalization.

Although we determined that the DRM-associated population of the CD-MPR is relatively small (~10%) at steady state, this represents nevertheless a significant fraction of the receptor considering its fast cycling through endosomes (Johnson and Kornfeld, 1992). Furthermore, the amount of the CD-MPR associated with DRMs is similar to the one that has been found in a previous study by quantitative electron microscopy as the endosomal population of the CD-MPR at steady state in

HepG2 cells (10%-20%) (Klumperman *et al.*, 1993). S-palmitoylation of the CD-MPR has been shown previously to happen at the endosomal level and at the PM (Stockli and Rohrer, 2004). Therefore, we assume that S-palmitoylation allows the endosomal CD-MPR population to associate with DRM sub-domains located in the early endosomes and that this association protects the CD-MPR from trafficking towards the lysosomes where it would be rapidly degraded. This is in good agreement with previous work emphasizing the importance of the proper lipid organization of the endosomal compartment for the proper trafficking of various proteins. For example, a U18666A treatment which disrupts the endosomal lipid composition by inducing a cholesterol accumulation in late endosomes (Liscum and Faust, 1989) leads to the re-localization of CI-MPR in a Lamp1 positive late endosomal/lysosomal compartment in skin fibroblasts (Kobayashi *et al.*, 1999). Similarly, Anthrax toxin (Abrami *et al.*, 2003) and Vesicular Stomatitis Virus (Le Blanc *et al.*, 2005) which both need to traffic to the late endosomal compartment for cytosolic translocation remain trapped in the endosomal compartment upon U18666A treatment in baby hamster kidney cell (Sobo *et al.*, 2007).

It is also very interesting that the internalization mutant of the CD-MPR which is trapped at the PM is palmitoylated at a much higher level than the wild type CD-MPR (Stockli and Rohrer, 2004) but nevertheless associates only very weakly with PM DRMs. This result could be explained by the difference of lipid composition between the PM DRMs (Brown and London, 1998) and the endosomal DRMs. (Brown and London, 1998; Kobayashi *et al.*, 2002). The association of the CD-MPR with endosomal DRMs might be enhanced by an interacting protein localized in endosomal DRMs which could stabilize the palmitoylated CD-MPR in those specific DRMs. Indeed, interactions between a fatty acyl binding protein and an S-palmitoylated protein have been reported before (i.e. CD36) (Spitsberg *et al.*, 1995;

Tao *et al.*, 1996) although the role of S-palmitoylation in this interaction has not been explored yet.

If S-palmitoylation enhances the proper localization of the CD-MPR on a subset of membranes within the endosomal compartment, this might explain why the palmitoylation deficient mutant of the CD-MPR is down-regulated through lysosomal degradation.

Previous work suggested that the failure of palmitoylation deficient CD-MPR mutants to efficiently recycle from endosomes back to the TGN ultimately results in its "passive" mis-trafficking to the lysosomal compartment (Schweizer *et al.*, 1996). However, the CD-MPR can be ubiquitinated and one of the well established roles of ubiquitination in the context of integral membrane proteins is lysosomal targeting. The CD-MPR is a very stable protein which exhibits a half-life of more than 48 hours (Rohrer *et al.*, 1995) which excludes the possibility that a large amount of the receptor is modified with ubiquitin under physiological conditions specifically to target it to lysosomes. Nevertheless, a possible ubiquitin-mediated degradation of the CD-MPR could explain the observed instability of some CD-MPR mutants. Breuer et al. (1998) observed that CD-MPR mutants with deletions of a short amino acid stretch within their cytoplasmic tail display an abnormal shortened half-life which could be extended by lactacystin, an inhibitor of ubiquitin mediated degradation. Thus, ubiquitination of the CD-MPR might be a "quality control" mechanism which mediates the degradation of damaged (and thus potentially mis-localized) receptors similar to the mechanism recently proposed by Emr and colleagues (2008) for specific transporters in yeast. Such a quality control mechanism would make much sense in the context of a house-keeping protein exhibiting an extensive half-life such as the CD-MPR. Indeed, the failure to discard

physiologically very stable house-keeping proteins that are damaged would ultimately lead to the accumulation of defective proteins of this kind.

All those results let us to propose a model for the endosomal trafficking of the CD-MPR (Fig. 6). Upon delivery and release of its ligand within the endosomal compartment, the CD-MPR is quickly S-palmitoylated. S-palmitoylation allows the CD-MPR to associate with endosomal DRMs where the trafficking machinery responsible for the retrieval of the CD-MPR to the TGN is located (Fig.6A). Non functional CD-MPRs are incorporated in intra-luminal vesicles of nascent endosomal multi-vesicular bodies for subsequent lysosomal degradation in an ubiquitin dependent manner (Fig.6B). A minority of the CD-MPR pool which escaped its retrieval from the early endosomes (EE) to the TGN and localizes to the late endosomes (LE) in an ubiquitin independent manner can undergo a Rab9-dependent LE to TGN retrograde transport to avoid an unspecific lysosomal degradation (Diaz *et al.*, 1997) (Fig.6C). Finally, CD-MPR localized at the PM undergoes both ubiquitination and S-palmitoylation but those two modifications would be functional once the CD-MPR is delivered to the endosomal compartment after internalization from the PM (Fig.6D).

Altogether, the results presented in this work suggest that S-palmitoylation plays a role in the retrieval of the CD-MPR from the endosomes to the TGN by allowing its association with specialized early endosomal DRM sub-domains. Additionally, the ubiquitination of the CD-MPR suggests a potential role in a “quality control” mechanism for the degradation of mis-functional and/or mis-localized receptors.

Acknowledgement

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Figures

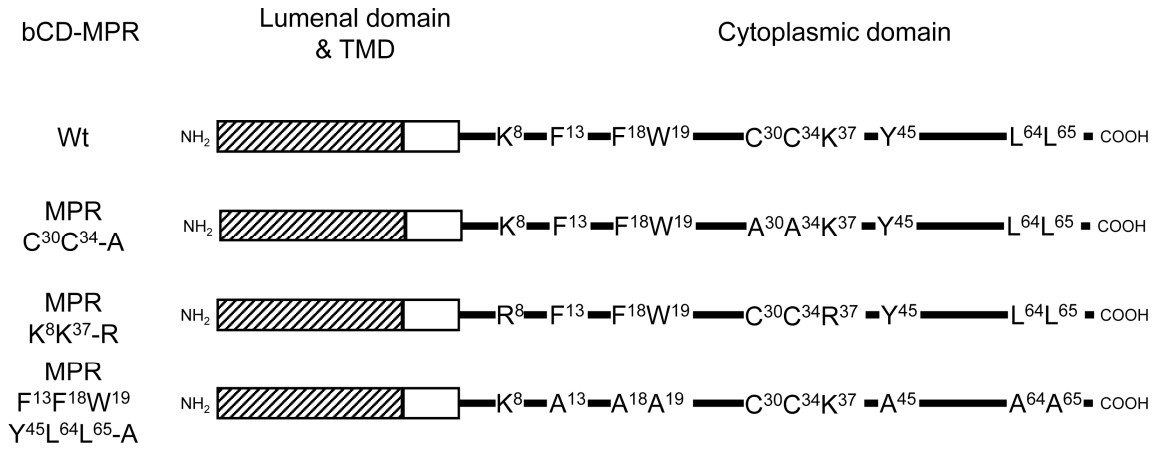

Figure 1: Schematic representation of the bovine CD-MPR constructs used in this work.

The hatched bars represent the luminal domain of the bovine CD-MPR (bCD-MPR) and the open bars the trans-membrane domain (TMD). The cytoplasmic domain is represented as a black line with selected amino acids indicated in single letter code. Wild type bCD-MPR (Wt); Palmitoylation-deficient bCD-MPR mutant (MPR $C^{30}C^{34}$-A); Ubiquitination-deficient bCD-MPR mutant (MPR $K^{8}K^{37}$-R); Internalization-deficient bCD-MPR mutant (MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A)

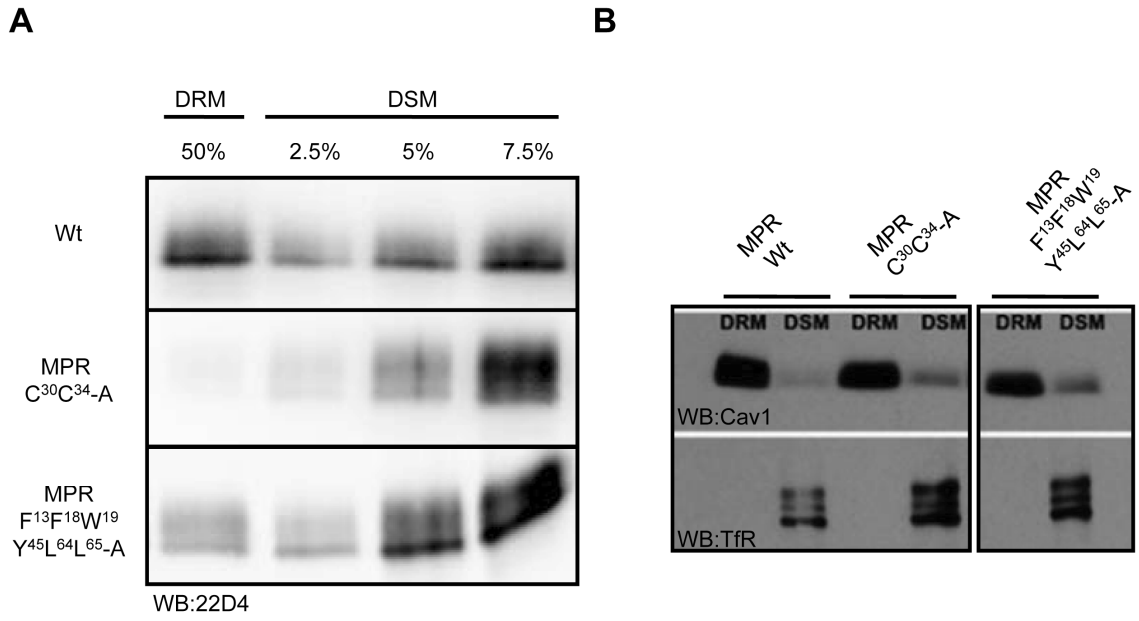

Figure 2: CD-MPR associates with detergent-resistant membranes.

Detergent-resistant membranes were extracted from HeLa cell lines stably transfected with different bCD-MPR mutants using cold 1%TRITON X-100. (A) 50% of the detergent-resistant membrane (DRM) fraction, followed by 2.5, 5 and 7.5% of the detergent-sensitive membrane (DSM) fraction of HeLa cell lines stably transfected with the specified bCD-MPR construct were probed by Western blot for the bCD-MPR. (B) 10% of the DRM and DSM fractions of HeLa cell lines stably transfected with the specified bCD-MPR construct were tested for caveolin-1 (Cav1) and transferrin receptor (TfR) by Western blot.

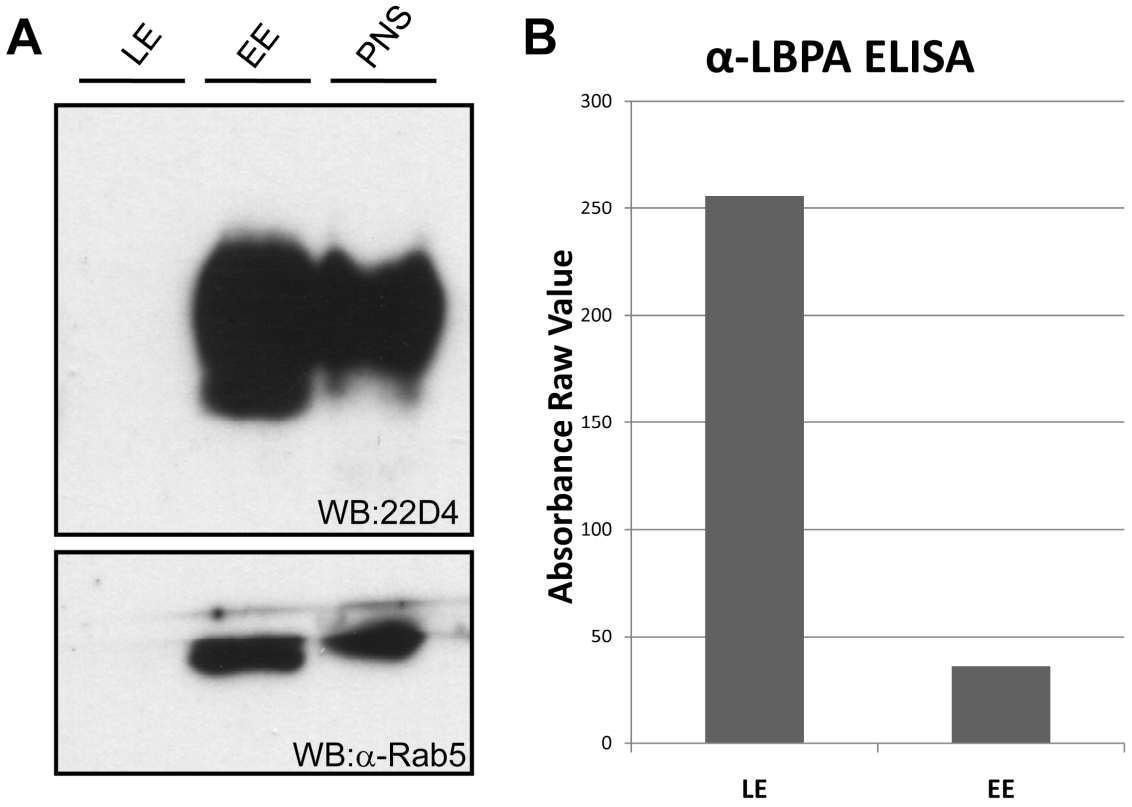

Figure 3: CD-MPR is not detectable in late endosomes.

Late endosomes were purified by a sucrose step gradient from BHK cells transiently transfected with a wild type bCD-MPR construct. (A) Late endosomal and early endosomal fractions were probed by Western blot for the bCD-MPR and rab5. (B) Late endosomal and early endosomal fractions were probed for LBPA by ELISA.

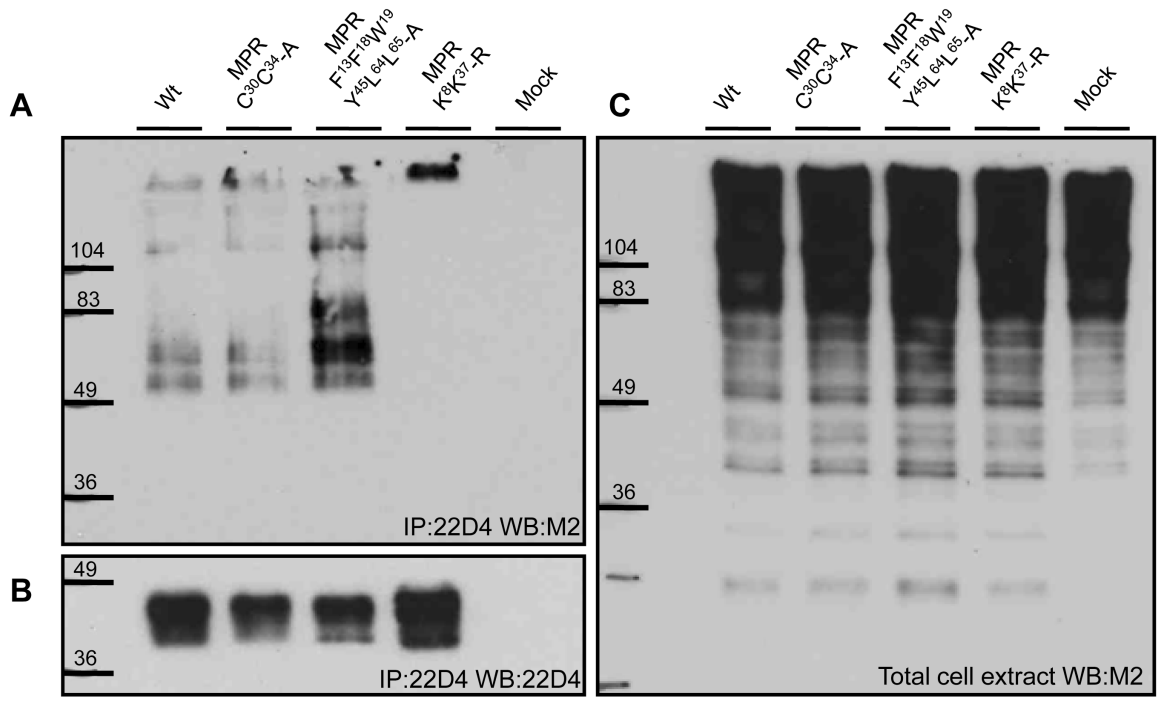

Figure 4: CD-MPR is ubiquitinated on its cytoplasmic domain.

HEK293 cells were co-transfected with a FLAG-Ubiquitin construct and different bCD-MPR constructs (Wt, MPR $C^{30}C^{34}$-A, MPR $K^{8}K^{37}$-R, MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A) as indicated. (A) Immunoprecipitated bCD-MPRs were probed for incorporated FLAG epitopes by Western blot. (B) After detection of the FLAG epitope, Western blot membranes were stripped and probed with 22D4 to assess bCD-MPR expression levels in the different samples. (C) Samples of the total cell extract were probed for FLAG-ubiquitin by Western blot.

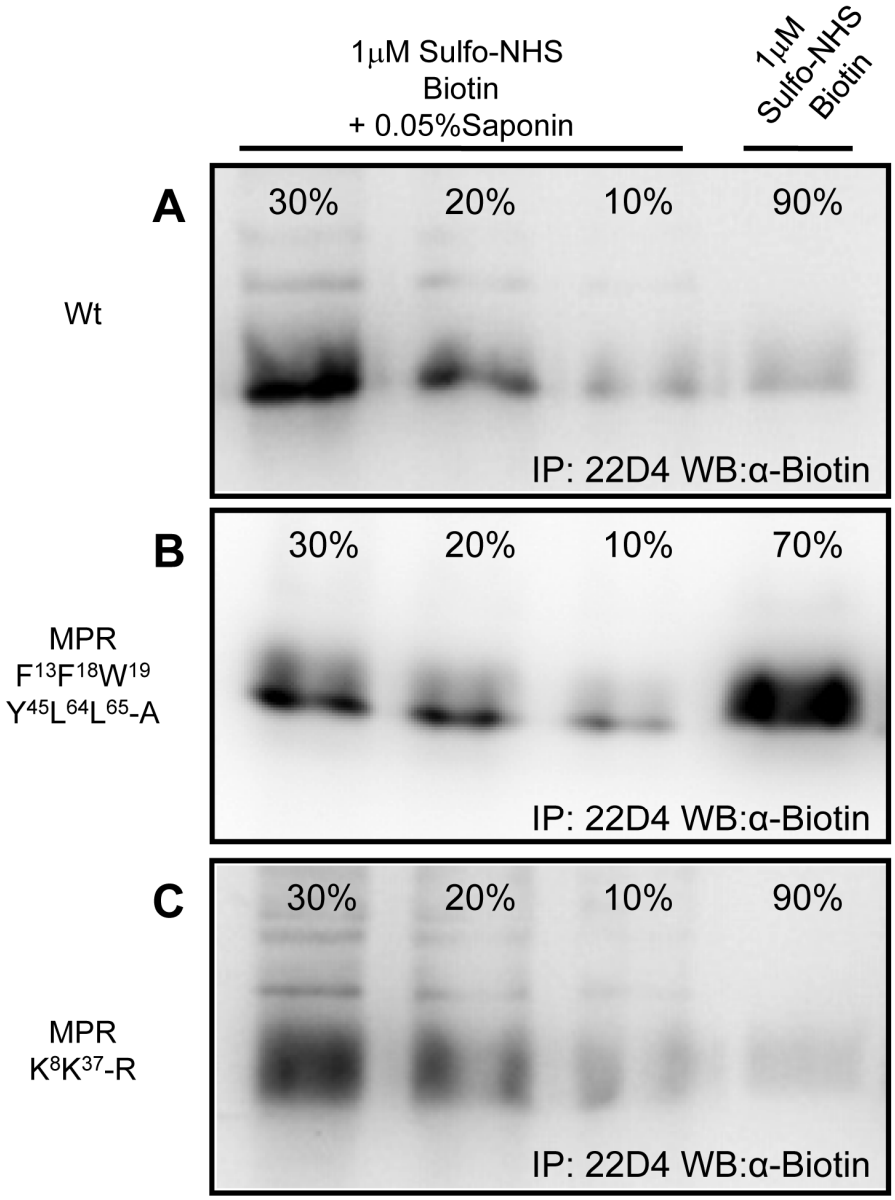

Figure 5: Ubiquitination-deficient CD-MPR mutant is not accumulating at the plasma membrane

Differential protein biotinylation assay was performed on HeLa cell lines stably transfected with different bCD-MPR constructs (Wt, MPR K^8K^{37}-R, MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A) as indicated. After immuno-precipitation, 30%, 20% and 10% of the sample treated in presence of 0.05% saponin followed by 90% (for A and C) or 70% (for B) of the sample treated without saponin were probed by Western blot for biotin. The HeLa cell line used for the assay was stably transfected with the wild-type bCD-MPR (Wt) in (A), the internalization-deficient bCD-MPR mutant (MPR $F^{13}F^{18}W^{19}Y^{45}L^{64}L^{65}$-A) in (B) and the ubiquitination-deficient bCD-MPR mutant (MPR K^8K^{37}-R) in (C)

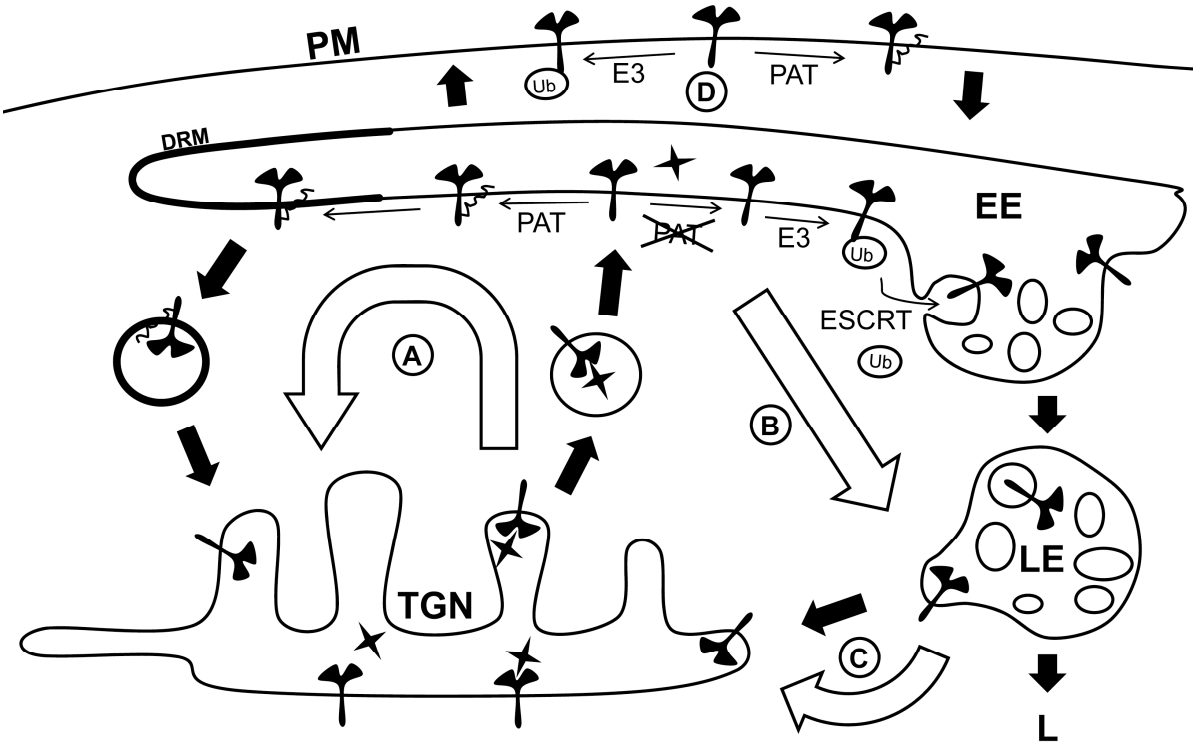

Figure 6: Model of CD-MPR intra-endosomal trafficking.

Upon arrival in the endosomal compartment, the CD-MPR is S-palmitoylated by a protein acyl transferase (PAT) activity. (A) The newly S-palmitoylated CD-MPR can then associate with endosomal DRMs for subsequent retrieval to the TGN. (B) Non functional CD-MPR is ubiquitinated and incorporated into endosomal intra-luminal vesicles for subsequent lysosomal targeting and degradation. (C) A minority of the CD-MPR pool which localized on the outer membrane of the late endosomes (LE) can undergo a Rab9-dependent late endosomes to TGN transport step. (D) CD-MPR localized at the PM is both S-palmitoylated and ubiquitinated. Legend: MVB: multi-vesicular bodies, PM: plasma membrane, TGN: trans-Golgi network, L: lysosomes.

Results

Part II

Identification of a restricted number of ZDHHC proteins as putative PAT of the cation-dependent mannose 6-phosphate receptor

Bruno G. H. Filippi[*]; Beat E. Schaub and Jack Rohrer[*‖]

[*]Institute of Physiology, University of Zurich, Zurich, Switzerland.

‖ Corresponding author: email: roja@zhaw.ch Present address: Institute for Biotechnology, Zurich University of Applied Sciences, Wadenswil, Switzerland

Manuscript in preparation

Summary

The cation dependent mannose 6-phosphate receptor constitutively cycles between the trans-Golgi network and the endosomes to deliver newly synthesized mannose 6-phosphate tagged lysosomal enzymes from the trans-Golgi network via endosomes to the lysosomal compartment. Sorting of the cation-dependent mannose 6-phosphate receptor within the endosomal compartment has been shown to require the S-palmitoylation of a cystein residue in its cytoplasmic tail. Interestingly, a protein acyl-transferase activity for the cation-dependent mannose 6-phosphate receptor has been found within the endosomes and at the plasma membrane of HeLa cells. Although the enzymology of S-palmitoylation is poorly characterized, several members of the ZDHHC protein family have been shown to be protein acyl transferases. The aim of this work is to characterize members of the ZDHHC protein family and to test the protein acyl transferase activity of ZDHHC proteins for the cation-dependent mannose 6-phosphate receptor. It is shown here that most of the ZDHHC proteins are expressed in HeLa cells and that individual ZDHHC proteins localize to different compartments throughout the secretory/endocytic pathway. In addition, a phylogenetic classification of ZDHHC genes was used to group the ZDHHC proteins into sub-classes. Surprisingly, the over-expression of individual ZDHHC proteins in HeLa cells did not alter the level of S-palmitoylation of the cation-dependent mannose 6-phosphate receptor to a large extend in despite of the identification of several ZDHHC proteins localizing to the endosomes and the plasma membrane. Overall, the expression profile of the ZDHHC genes, the intracellular localization of the ZDHHC proteins and the phylogenetic data presented in this work indicate a restricted number of ZDHHC proteins as putative protein acyl transferase of the cation-dependent mannose 6-phosphate receptor.

Introduction

The cation dependent mannose 6-phosphate receptor (CD-MPR) is a type I integral transmembrane protein which sorts newly synthesized lysosomal enzymes tagged with a mannose 6-phosphate residue from the trans-Golgi network (TGN) to the lysosomes via the endosomes (Dahms and Hancock, 2002). For its sorting within endosomes, the CD-MPR requires the S-palmitoylation of its cytoplasmic tail (Schweizer *et al.*, 1996). The abolishment of this modification results in the mis-routing of the receptor to lysosomes and in the loss of its ability to sort lysosomal enzymes (Schweizer *et al.*, 1996). S-palmitoylation is a common protein lipidation which consists of a reversible attachment of a 16-carbon saturated fatty acid to a protein through a thio-ester linkage (Linder and Deschenes, 2003). Similarly to other protein lipidations (e.g. GPI-anchors, N-myristoylation, prenylation…), it modulates the hydrophobicity of the proteins it is attached to. Thus, such a modification usually promotes the association of cytosolic proteins with lipid bilayers and/or the re-localization of cytosolic and membrane proteins into specialized membrane sub-domains (Linder and Deschenes, 2007). However, S-palmitoylation differs from all other protein lipidations by being reversible through the action of thio-esterase activities (Yeh *et al.*, 1999; Duncan and Gilman, 2002) and this reversibility makes S-palmitoylation a potent regulator of proteins which rely on their intracellular localization to fulfill their biological function. Such palmitoylated proteins are playing important roles in numerous biological mechanisms such as immunology (e.g: CD4 (Fragoso *et al.*, 2003), LAT (Zhang *et al.*, 1998), CD8 (Arcaro *et al.*, 2000)), cell cycle (e.g. Ras (Cadwallader *et al.*, 1994), ERBIN (Izawa *et al.*, 2008)) or synaptic plasticity (Washbourne, 2004). In previous work, the CD-MPR has been shown to be S-palmitoylated within endosomes and at the plasma membrane (PM) in HeLa cells but a protein acyl transferase (PAT) for the CD-MPR has not been identified

yet (Stockli and Rohrer, 2004). Interestingly, several members of the ZDHHC protein family have been found to be enzymes that can catalyze the S-palmitoylation of substrate proteins. Members of the ZDHHC protein family are multipass integral transmembrane proteins which have a distinctive protein motif (ZDHHC motif) that has been implicated in the catalysis of S-palmitoylation. For recent reviews, see (Tsutsumi *et al.*, 2008; Iwanaga *et al.*, 2009). The human genome contains twenty-three ZDHHC genes and the RefSeq project at the NCBI (Pruitt *et al.*, 2007) identified up to thirty-six ZDHHC mRNAs potentially encoding thirty-one ZDHHC proteins (table 1). Therefore, ZDHHC proteins form a large protein family. However, most members of the ZDHHC protein family are still not or poorly characterized.

In this work, the endogenous expression of ZDHHC proteins was determined as well as the intracellular localization of myc-tagged ZDHHC proteins. In addition, the phylogeny of the human ZDHHC genes was investigated by comparing their intron/exon structure. Finally, the effect of the over-expression of various ZDHHC proteins on the level of S-palmitoylation of the CD-MPR in HeLa cells was tested. Using these methods, we were able to indicate a restricted number of ZDHHC proteins as putative PAT of the CD-MPR.

Materials and methods

Materials

Chemicals were from AppliChem (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, U.S.A.). Protein inhibitor cocktail (SigmaPIC) was obtained from Sigma-Aldrich. Nitrocellulose was from Whatman PLC (Florham Park, U.S.A.). Dulbecco's modified Eagle medium (DMEM) and bovine fetal calf serum (FCS) were from Invitrogen (Carlsbad, U.S.A.). Disposable plastic ware and cell culture dishes were from TPP (Trasadingen, Switzerland), VWR (Wien, Austria), Greiner Bio One (Monroe, U.S.A.) or Bioswisstec (Schaffhausen, Switzerland). Low-fat dry milk powder was from Coop (Basel, Switzerland).

General solutions

Unless specified differently, solutions are made in ddH_2O. Phosphate buffer saline (PBS) (3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, pH 7.4); stripping solution (0.2 M glycine, 2% SDS, pH 2.5); sample buffer (SB) (37.5 mM Tris-HCl, 8% SDS, 10% glycerol, 0.003% bromophenol blue, 50 mM DTT, pH 6.5); 500× PIC (5mg/ml benzamidine, and 1µg/ml of pepstatin A, leupeptin, antipain, and chymostatin in 40% dimethylsulfoxide-60% ethanol)

Tissue culture and transfection

HeLa cells were grown in DMEM + 10% FCS at 37℃ in a 5% CO_2 atmosphere. Transfections were performed using the "cell line Nucleofector® Kit R" from Amaxa (Cologne, Germany) accordingly to the instructions of the manufacturer. Briefly, one million of freshly trypsinized HeLa cells were electroporated using 2 µg of DNA and subsequently seeded in appropriate cell culture dishes.

Antibodies

The monoclonal antibody 22D4 purified from hybridoma supernatant was used against the bovine CD-MPR (Messner, 1993). When the monoclonal antibody 22D4

was used, all reducing agents were omitted. The monoclonal 9e10 purified from hybridoma supernatant was used against myc-tagged proteins (Evan *et al.*, 1985); the anti-biotin-HRP antibody was a polyclonal antibody from Sigma-Aldrich; the sheep anti-mouse IgG HRP antibody was "ECL Anti-mouse IgG Horseradish peroxidase linked whole antibody from sheep" from GE healthcare (Chalfont St.Giles, UK); the anti-mouse immuno-globulin Alexa 568 antibody was from Invitrogen (Carlsbad, U.S.A)

SDS-PAGE, Western blotting and immuno-detection

SDS-PAGE electrophoresis of samples was performed according to Laemmli (1970). Western blot was performed according to Towbin et al (1979). Unless differently specified, all immuno-detection steps were performed at room temperature. Prior to immuno-detection, the nitro-cellulose membrane resulting from a Western blot was blocked with a solution of 3% milk (w/v) in PBS (MPBS) for 1 hr and subsequently rinsed three times for thirty seconds with PBS. Immuno-detection of bovine CD-MPR (bCD-MPR) was performed with the monoclonal antibody 22D4 at a concentration of 1µg/ml in MPBS for one hour. After the incubation, the nitro-cellulose membrane was washed three times for five minutes in MPBS and the primary antibody was detected with a 1/2000 dilution of a polyclonal sheep antibody to mouse IgG antibody coupled with HRP in MPBS for one hour. Finally, the membrane was washed three times ten minutes in PBS. Immuno-detection of biotin was performed with over-night incubation in a 1/500 dilution of polyclonal anti-biotin antibody coupled to HRP in a solution of 3% BSA (w/v) in PBS at 4℃. After the incubation, the nitro-cellulose membrane was washed three times twenty minutes in PBS. Unless differently specified, the final detection of HRP-conjugated immuno-globulins adsorbed on the nitro-cellulose membranes was done by using a chemiluminescent substrate (Supersignal West Pico, Pierce, Rockford, U.S.A)

according to manufacturer's instructions and subsequently exposing the nitro-cellulose membrane to an autoradiography film (Kodak biomax light film, Sigma-Aldrich).

Acyl-Biotin exchange assay

HeLa cells were grown to ninety percents confluence in a 3.5 cm tissue culture well. Cells were washed twice with PBS and directly solubilized in the well by adding 1.3 mL of lysis buffer (PBS, 0.5% Triton X-100, 50 mM NEM, 1 mM EDTA, SigmaPIC, pH7) and gentle shaking for ten minutes at 4℃. The lysate was then passed five times through a syringe equipped with a G25 needle and transferred to an Eppendorf tube. After five additional minutes of solubilization on ice, the sample was centrifuged at 16.1 krcf for fifteen minutes at 4℃. The supernatant was collected and the pellet discarded. The sample was incubated two hours at 4℃ with 35 µl of proteinA-22D4 DMP crosslinked beads on an end over end shaker to immuno-precipitate the bCD-MPR. Subsequently, the beads were washed three times ten minutes with 0.1% Triton X-100 in PBS. S-palmitoyl groups were cleaved from the bCD-MPR and the newly freed thiol groups immediately labeled with biotin by incubating the beads in 100 µL of biotinylation buffer (1 M Hydroxylamine; 4 µM biotin-HPDP; 1 mM EDTA, pH 7.1) for forty-five minutes at room temperature. Finally, the beads were washed once with PBS and the bound proteins eluted at 95℃ for three minutes in 50 µl SB. Samples were re solved on a 10% SDS-PAGE, transferred onto a PVDF membrane by Western blot and biotin was immuno-detected. The PVDF membrane was then stripped by two incubations of fifteen minutes in stripping solution at 50℃, subsequently rinsed three times for one minute with PBS and blocked for one hour in MPBS at room temperature. Finally, the bCD-MPR was detected as described above. The intensity of the chemiluminescence signals emitted during the immuno-detection of biotin and bCD-

MPR were quantified using a DIANA III chemiluminescence detection system (Raytest, Straubenhardt, Germany).

Immuno-fluorescence

HeLa cells were grown on cover-slips to a density of approximately fifty percents. The cells were then washed with PBS and fixed by incubating the cover-slip fifteen minutes in a drop of PFA-PBS (3% paraformaldehyde in PBS). The cells were briefly washed with PBS-glycine (20 mM glycine in PBS) and permeabilized by an incubation of ten minutes in a solution of PBS-glycine-saponin (0.1% saponin and 20 mM glycine in PBS). Subsequently, the cells were incubated with the monoclonal antibody 9e10 at a concentration of 1 µg/ml in PBS-saponin (0.1% saponin in PBS) for thirty minutes, briefly washed four times in PBS-saponin, incubated for thirty minutes in a drop of PBS-saponin supplemented with 1 µg/ml DAPI and an anti-mouse immunoglobulin antibody coupled with Alexa 568 (Panchuk-Voloshina *et al.*, 1999), washed again four times in PBS-saponin and finally mounted on a glass slide using Prolong Gold anti-fade (Invitrogen, Carlsbad, U.S.A.). Samples were analyzed using an Axiovert 200M epifluorescence microscope from Zeiss (Oberkochen, Germany).

Total RNA extraction and cDNA synthesis

HeLa cells were grown in a 10 cm Petri dish to a density of approximately ninety percents. The cells were washed twice with ice cold PBS, scraped in 1 ml of ice cold PBS and the resulting suspension transferred in a 1.5 ml eppendorf tube. The cells were then sedimented by centrifuging the cell suspension for five minutes at 200 rcf at 4℃ and the resulting supernatant was discarded. Total RNA was extracted from the pelleted HeLa cells using TRI reagent (Sigma-Aldrich, St. Louis, U.S.A.) accordingly to the instructions of the manufacturer. The purified total RNA was then used as a template to generate the corresponding cDNA using the

SuperScript™ double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, U.S.A.) according to the instructions of the manufacturer.

Real time polymerase chain reaction

Every real time polymerase chain reaction (RT-PCR) samples had a total volume of 25 µl and was containing 50 ng of cDNA, 5 pg of each primer of a primer pair and 12.5 µl of SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich). The RT-PCR reactions were performed in triplicate using an Mx3000P QPCR machine (Stratagene, La Jolla, U.S.A.) operating a PCR program composed of an initial denaturation step (ten minutes at 95℃) followed by forty repeats of a sequence comprising a denaturation step (five seconds at 95°C) followed by an annealing step (variable time and temperature depending on the primer pair used in the RT-PCR reaction, see supplementary Fig.1 for details) and an elongation step (thirty seconds at 72℃). The total quantity of double-stranded DNA present in every sample was measured every cycle at the end of the annealing step. At the end of the forty cycles, a fusion curve of the double-stranded DNA present in every sample was produced by gradually heating every sample from 55℃ to 95℃ while constantly measuring the quantity of double-stranded DNA.

Results

Expression of ZDHHC genes in HeLa cells

The endogenous expression of the ZDHHC genes in HeLa cells was determined using real time polymerase chain reaction. Primer pairs have been successfully designed to detect twenty-four of the thirty-six ZDHHC mRNAs (table 2). Those primer pairs amplify a single specific DNA product only in the presence of their corresponding DNA template sequence. Total RNA has been extracted from HeLa cells and the corresponding cDNA generated. RT-PCRs were then performed using this cDNA sample as a template to detect the presence of the ZDHHC mRNAs in HeLa cells. Using this approach, most of the ZDHHC mRNAs tested were found to be transcribed in HeLa cells (ZDHHC#2, ZDHHC#3_1, ZDHHC#3_3, ZDHHC#4, ZDHHC#5, ZDHHC#6, ZDHHC#8, ZDHHC#9, ZDHHC#13_1, ZDHHC#13_2; ZDHHC#15_1, ZDHHC#15_2; ZDHHC#17, ZDHHC#18, ZDHHC#20 and ZDHHC#24; Table 2). However, the mRNAs encoding ZDHHC#1, ZDHHC#12, ZDHHC#19 and ZDHHC#21 were not detected in Hela cells which indicates that they are only transcribed in specialized cells (table 2). Although the expression of six ZDHHC genes (ZDHHC#7, ZDHHC#11, ZDHHC#14, ZDHHC#16, ZDHHC#22 and ZDHHC#23) and the presence of mRNAs encoding ZDHHC#3_2 remain to be tested (table 2), this result suggests that most of the ZDHHC proteins are expressed in HeLa.

Intracellular localizations of ZDHHC proteins in HeLa cells

To further characterize members of the ZDHHC protein family, the intracellular localization of the different ZDHHC proteins was analyzed. The cDNA sequences encoding sixteen of the thirty-one ZDHHC proteins were obtained from the I.M.A.G.E. consortium (Lennon *et al.*, 1996). The cDNA of the ZDHHC sequences were fused on their 5' end with a DNA sequence encoding a Myc-tag peptide and

sub-cloned into a mammalian expression vector. The resulting constructs were then individually transfected into HeLa cells and the intracellular localization of the proteins was determined by immuno-fluorescence microscopy. As shown in figure 2, fourteen Myc-ZDHHC constructs produced detectable proteins which had various intracellular localizations throughout the secretory/endocytic pathway. Myc-ZDHHC#4 and Myc-ZDHHC#24 had a staining pattern typical for ER localization whereas Myc-ZDHHC#3, Myc-ZDHHC#13, Myc-ZDHHC#16, Myc-ZDHHC#17 and Myc-ZDHHC#21 were localized within the Golgi apparatus. In contrast, Myc-ZDHHC#1, Myc-ZDHHC#2, Myc-ZDHHC#7, Myc-ZDHHC#11, Myc-ZDHHC#15, Myc-ZDHHC#18 and Myc-ZDHHC#20 had a peripherally punctuated staining pattern indicating a localization in endosomes and/or the plasma membrane localization. Finally, ZDHHC#6 contains the same ER recycling signal –KKXX (Jackson *et al.*, 1990) than ZDHHC#4 and was therefore not tested because it is expected to be localized in the ER as well. The intracellular localization of the Myc-ZDHHC proteins determined using this approach is summarized in table 3.

Phylogeny of the ZDHHC gene family

Several ZDHHC proteins have a high level of primary sequence identity; most notably within a forty-four amino acid region that encompasses the ZDHHC motif (Fig. 1A). This observation led several authors to perform phylogenetic analyses of the ZDHHC protein family (Mitchell *et al.*, 2006; Tsutsumi *et al.*, 2008; Iwanaga *et al.*, 2009). However, those phylogenetic studies were all made on the basis of protein sequences alignment and their results show discrepancies. Intron positions are potent markers to perform phylogenetic analyses (Tijet *et al.*, 2001; Krauss *et al.*, 2008) because similarities in the intron/exon organization of genes indicate that they evolved from the duplication of a common ancestral gene already having such a intron/exon organization (Rogozin *et al.*, 2005). Thus, we analyzed the phylogeny

of the ZDHHC genes by comparing their intron/exon organization. The human genomic contigs containing the ZDHHC genes were retrieved from the NCBI database and the exons encoding the ZDHHC motif of the ZDHHC proteins were compared. As shown in figure 1B, fifteen ZDHHC genes can be grouped into six different groups on the basis of the conservation of the intron encoding the DHHC tetra-peptide; whereas the last eight remaining ZDHHC genes (ZDHHC#4, ZDHHC#6, ZDHHC#12, ZDHHC#16, ZDHHC#21, ZDHHC#22, ZDHHC#23 and ZDHHC#24) do not share their intron/exon organizations with any other ZDHHC genes and form a seventh and last group (data not shown). This result is convincing evidence that the ZDHHC genes classified in the group one to six evolved to diverge from duplications of ancestral ZDHHC genes.

Analysis of the PAT activity of ZDHHC proteins for the CD-MPR

During the work presented above, we identified six ZDHHC proteins (ZDHHC#2, ZDHHC#7_1, ZDHHC#11, ZDHHC#15_1, ZDHHC#18 and ZDHHC#20) expressed in HeLa cells that are localized at the PM and in peripheral endosomes as it was determined for the PAT activity of the CD-MPR by Stockli and colleagues (2004). Therefore, the effect of the over-expression of those six ZDHHC proteins on the level of S-palmitoylation of the CD-MPR was tested in HeLa cells. Such an approach based on over-expression previously allowed the identification of several ZDHHC proteins as the PATs of different S-palmitoylated proteins (Fukata *et al.*, 2004; Swarthout *et al.*, 2005; McCormick *et al.*, 2008; Tsutsumi *et al.*, 2009). To achieve this aim, a previously described protein S-palmitoylation detection assay (Drisdel and Green, 2004) was improved to determine in a semi-quantitative manner the level of S-palmitoylation of the CD-MPR (referred to as the acyl-biotin exchange (ABE) assay). Briefly, the CD-MPR is immunoprecipitated from a protein extract and treated with neutral hydroxylamine which specifically cleaves the

thioester bond of S-palmitoylated cystein residues and leaves a thiol group that is subsequently modified with HPDP-biotin. The sample is then resolved by SDS-PAGE, blotted on a PVDF membrane by Western blot and the biotin as well as the total CD-MPR are immuno-detected. The degree of S-palmitoylation of the CD-MPR was determined by normalizing the signal for the immuno-detection of biotin with the value for the signal of the total CD-MPR. To validate this approach, our ABE assay was used on HeLa cell lines stably expressing either a palmitoylation deficient CD-MPR mutant (MPR CC-A) or a hyper-palmitoylated internalization deficient CD-MPR mutant (MPR FFWYLL-A) (Stockli and Rohrer, 2004). As a control, the ABE assay was also used with a HeLa cell line stably expressing an ubiquitination deficient mutant form of the CD-MPR (MPR KK-R) that is not expected to have a different level of S-palmitoylation compared to the wild type form of the receptor (Fig. 3A). Additionally, it was tested if the incorporation of biotin into the CD-MPR occurs only if the sample is treated with hydroxylamine. A comparison of the biotin immuno-detection signals obtained in presence or in absence of the hydroxylamine treatment shows that this approach specifically detects palmitoylated cystein residues (Fig. 3A). As expected, the level of S-palmitoylation determined for the palmitoylation deficient mutant form of the CD-MPR is only approximately 3% of the value determined for the wild type form of the receptor. The level of S-palmitoylation determined for the hyper-palmitoylated mutant form of the CD-MPR (MPR FFWYLL-A) is approximately 260% of the value determined for the wild type form of the receptor (Fig. 3B). Finally, the level of S-palmitoylation determined for the ubiquitination deficient mutant form of the receptor was not significantly different from the level determined for the wild type form of the receptor (Fig. 3B). All together, the results validate our palmitoylation assay which allows to compare the level of S-palmitoylation of various forms of the CD-MPR.

Thus, we used this assay to test the effect of the over-expression of the ZDHHC proteins that were found to localize in peripheral endosomes (ZDHHC#2, ZDHHC#7_1, ZDHHC#11, ZDHHC#15_1, ZDHHC#18 and ZDHHC#20). If the PAT of the CD-MPR would be among those ZDHHC proteins, we would expect a higher level of S-palmitoylation of the CD-MPR when the correct PAT is over-expressed. The Myc-ZDHHC constructs were individually transfected into a HeLa cell line stably expressing the wild-type form of the bovine CD-MPR and the level of S-palmitoylation of the receptor was determined. For every experiment, the efficiency of individual transfections was determined by detecting myc-tagged ZDHHC proteins using immuno-fluorescence microscopy. The transfection efficiency of the different ZDHHC constructs was ranging from forty to eighty percent, whereas the transfection efficiency of a given construct was comparable between experiments with a variation of about 5% (data not shown). The transfection of ZDHHC#7_1 did lead to a slightly higher level of palmitoylation on average (120±28%, Fig. 4A) but this value is associated with a large standard error. However, the over-expression of the five other ZDHHC proteins did not alter the level of S-palmitoylation of the CD-MPR (ZDHHC#2, 101±28%; ZDHHC#11, 104±9%; ZDHHC#15_1, 110±21%; ZDHHC#18, 93±21%; ZDHHC#20, 112.5±27%; Fig. 4A). Since the over-expression of the ZDHHC proteins that localize in endosomes did not strongly alter the degree of S-palmitoylation of the CD-MPR, we repeated this experiment with the ZDHHC proteins which localize in the Golgi apparatus (ZDHHC#3_1, ZDHHC#13_1, ZDHHC#16_1, ZDHHC#17, ZDHHC#21) and with ZDHHC#24 which localizes in the ER as a control. The over-expression of two ZDHHC proteins did induce a slightly higher level of S-palmitoylation of the CD-MPR (ZDHHC#13_1, 124±37%; ZDHHC#16_1, 126±44%; Fig. 4B) but again these values are associated to a large standard error. On the other hand, the other ZDHHC proteins did not alter the level

of S-palmitoylation of the CD-MPR (ZDHHC#3_1, 94.5±6%; ZDHHC#17, 104%; ZDHHC#21, 114%; ZDHHC#24, 104±9%; Fig. 4B).

Discussion

The results show that most of the ZDHHC proteins are expressed in HeLa cells but individual ZDHHC proteins localize to different compartments throughout the secretory/endocytosis pathway. Additionally, the phylogenetic relationship between the human ZDHHC genes was studied on the basis of their intron/exon structure. The combination of all those results and the knowledge from previous experiments that the PAT activity cycles between the endosomes and the plasma membrane (Stockli and Rohrer, 2004) led to the identification of a restricted number of ZDHHC proteins which are good candidates to be the PAT(s) of the CD-MPR. However, none of the ZDHHC proteins tested in this work display a strongly elevated PAT activity for the CD-MPR.

The intracellular localization in HEK 293T cells of several ZDHHC proteins was determined by Ohno and colleagues (2006). Surprisingly, the results presented in their work concerning the intracellular localization of the ZDHHC proteins are strikingly different from ours. Most notably, Ohno and colleagues found twelve out of twenty-two tested ZDHHC proteins localizing in the ER but only two of those ZDHHC proteins (ZDHHC#4 and ZDHHC#6) have a typical –KKXX ER recycling signal (Jackson *et al.*, 1990). This result might arise from the accumulation of misfolded ZDHHC proteins in the ER potentially due to the N-terminal tag used by Ohno and colleagues (His_6-Myc or 3×FLAG). In our work, the ZDHHC proteins were N-terminally tagged with a single Myc peptide which is only six amino acids smaller but this might have made the difference because only two ZDHHC proteins, ZDHHC#24 and ZDHHC#4, were found to be localized in the ER. Moreover, the ER

localization of ZDHHC#4 appears physiological since it is one of the ZDHHC proteins bearing a typical –KKXX ER recycling signal. Therefore our results might represent the physiological intracellular localization of the ZDHHC proteins. The intracellular localization of fourteen out of the thirty-one predicted ZDHHC proteins has been determined in this work. Members of the ZDHHC protein family seem to localize to three main intracellular localizations. The first of these intracellular localizations is the endosomal and plasma membrane system where seven ZDHHC proteins have been found (ZDHHC#1, ZDHHC#2, ZDHHC#7, ZDHHC#11, ZDHHC#15, ZDHHC#18 and ZDHHC#20, Fig. 2). The second of these intracellular localizations is the Golgi apparatus in which five ZDHHC proteins have been observed. (ZDHHC#3, ZDHHC#13, ZDHHC#16, ZDHHC#17 and ZDHHC#21; Fig. 2). Finally, two ZDHHC proteins were found in the endoplasmic reticulum (ZDHHC#4 and ZDHHC#24; Fig. 2). It would be interesting to assay the intracellular localization of the seventeen remaining ZDHHC proteins. However, seven of these seventeen ZDHHC proteins are isoforms of ZDHHC proteins which intracellular localization has been determined in this work and it is therefore likely that protein isoforms have a similar intracellular localization. Consequently, ZDHHC#3_2, ZDHHC#3_3, ZDHHC#13_2, ZDHHC#16_2 and ZDHHC#16_3 might localize in the Golgi apparatus and ZDHHC#7_2 and ZDHHC#15_2 in peripheral endosomes. This result clearly demonstrates that the members of the ZDHHC protein family can potentially catalyze the S-palmitoylation of substrate proteins within the entire secretory/endocytic pathway.

In order to further characterize the ZDHHC protein family, a phylogenetic study of the ZDHHC gene family was performed in this work. Phylogenetic studies of the ZDHHC gene family were made in previous work (Mitchell *et al.*, 2006; Tsutsumi *et al.*, 2008; Iwanaga *et al.*, 2009). However, these studies were performed using

approaches based on the alignment of primary protein sequences. The result of this approach varies depending on multiple parameters such as the method used to align the primary protein sequences, the distance correction method used, the tree construction method used and the portion of the primary protein sequence used to perform the alignment. For an extensive description of the parameters to consider when performing phylogenetic analyses using sequence alignments, see Nei (1996) and Kelchner & Thomas (2007). Consequently, the outcomes of such analyses bear an intrinsic uncertainty and variability. The comparison of intron/exon structures of genes is an additional phylogenetic tool to trace the gene history (Tijet *et al.*, 2001; Krauss *et al.*, 2008). The intron/exon organization of the ZDHHC genes shows that fifteen of the twenty-three human ZDHHC genes can be grouped in six different groups in which the genes share identical introns (Fig 1B). Overall, the outcome of this analysis is in good agreement with the results of the previous phylogenetic studies of the ZDHHC family and confirms a close evolutionary linkage between ZDHHC#2, ZDHHC#15 and ZDHHC#20 as well as between ZDHHC#9, ZDHHC#14 and ZDHHC#18. A similar observation can be made for ZDHHC#3 and ZDHHC#7 and for ZDHHC#1 and ZDHHC#11. However, unlike previous studies, this approach reveals that ZDHHC#13 and ZDHHC#17 evolved from the duplication of a common ancestral gene and that ZDHHC#19 is phylogenetically linked to ZDHHC#5 and ZDHHC#8. Also, the ZDHHC genes that are not included in one of the six groups presented on the figure 1B (ZDHHC#4, ZDHHC#6, ZDHHC#12, ZDHHC#16, ZDHHC#21, ZDHHC#22, ZDHHC#23 and ZDHHC#24) have original intron/exon structures and this observation is a strong indication that they diverged a long time ago from the other ZDHHC genes. Interestingly, some ZDHHC proteins that are classified in the same phylogenetic group have been previously shown to have similar protein substrate specificities. For example, ZDHHC#13 and ZDHHC#17

which can palmitoylate huntingtin (Huang *et al.*, 2009) are both in the phylogenetic group 2 (Fig 1B). Similarly, ZDHHC#9 and ZDHHC#18 which can palmitoylate H-Ras (Fukata *et al.*, 2004; Swarthout *et al.*, 2005) are in the phylogenetic group 5 and ZDHHC#2 and ZDHHC#15 which can palmitoylate PSD-95 (Fukata *et al.*, 2004) are both in the phylogenetic group 1 (Fig 1B). Finally, ZDHHC#3 and ZDHHC#7 which also have similarities in their substrate specificities (Fang *et al.*, 2006; Tsutsumi *et al.*, 2009) are also in the same phylogenetic group (Fig 1B). Consequently, this phylogenetic classification might help to define groups of ZDHHC proteins having overlapping protein substrate specificities, and might provide important indications to determine the protein substrate specificities of ZDHHC proteins that have not been characterized so far. For example, ZDHHC#14 which is closely related to ZDHHC#9 and ZDHHC#18 might also be a PAT for the H-Ras and N-Ras or, alternatively, for another small GTPase similar to H-Ras. McCormick and colleagues (2008) provided good evidence that ZDHHC#15 is implicated in the S-palmitoylation of the CI-MPR and sortilin, two proteins involved in the sorting of lysosomal proteins similarly to the CD-MPR (Braulke and Bonifacino, 2008). Thus, according to the phylogeny of the ZDHHC protein family, ZDHHC#2, ZDHHC#15 or ZDHHC#20 appear as prime candidates to be PATs for the CD-MPR.

The overlapping protein substrate specificities of ZDHHC proteins prevent attempts to study the biological role of the ZDHHC proteins using approaches which take advantage of a knock-down of their expression (e.g. siRNA studies). Indeed, if several ZDHHC proteins are palmitoyl acyl-transferases for a same substrate protein, then the down-regulation of one of those ZDHHC proteins is not expected to result in a significant alteration of the level of S-palmitoylation of the substrate protein. Therefore, the information provided by the phylogenetic classification

established in this work might also help to design down-regulation experiments by indicating which ZDHHC proteins might have overlapping protein substrate specificities.

Despite the identification of several ZDHHC proteins expressed in HeLa and that some localize in the endosomes and at the PM, we did not identify a ZDHHC protein with a strong PAT activity for the CD-MPR using an over-expression approach. This result might be due to several reasons. The PAT of the CD-MPR could be among the ZDHHC proteins that were not tested in this work. The number of ZDHHC proteins tested in this work was restricted due to the limited number of full-length, wild-type ZDHHC cDNA that was available from the I.M.A.G.E. consortium. However, several of the ZDHHC proteins that were not tested (ZDHHC#3_2, ZDHHC#3_3, ZDHHC#7_2, ZDHHC#13_2, ZDHHC#15_2, ZDHHC#16_2 and ZDHHC#16_3) are just isoforms of ZDHHC proteins that have been tested in this work and their over-expression would probably not increase the level of S-palmitoylation of the CD-MPR either. In addition, ZDHHC#4 is localizing in the endoplasmic reticulum and ZDHHC#6 is expected to localize in the endoplasmic reticulum as well since it contains a C-terminal –KKXX ER recycling motif. Therefore, ZDHHC#4 and ZDHHC#6 cannot be the PAT of the CD-MPR since that activity has been shown to cycle between the plasma membrane and the endosomes (Stockli and Rohrer, 2004). The same exclusion criteria, localization in a different organelle than endosomes and plasma membrane, also applies for the ZDHHC#9 protein which has been previously found in the Golgi apparatus by Swarthout and colleagues (2005). Finally, ZDHHC#1, ZDHHC#12, ZDHHC#19 and ZDHHC#21 are not expressed in HeLa cells and therefore cannot be the PAT of the CD-MPR since the receptor is S-palmitoylated in HeLa cells (Stockli and Rohrer, 2004). Consequently, only six ZDHHC proteins that have not been tested in this

work (ZDHHC#5, ZDHHC#8, ZDHHC#14_1, ZDHHC#14_2, ZDHHC#22 and ZDHHC#23) are still potential candidates to be a PAT for the CD-MPR and it would be interesting to test the effect of their over-expression on the level of S-palmitoylation of the CD-MPR.

An alternative explanation that none of the ZDHHC proteins led to a higher level of S-palmitoylation of the CD-MPR is that the over-expression of the ZDHHC protein which is a PAT for the CD-MPR might require an additional co-factor. For instance, ZDHHC#9 requires GCP16, a Golgi-localized protein (Ohta *et al.*, 2003) to catalyze the S-palmitoylation of H-Ras and N-Ras (Swarthout *et al.*, 2005). However, up to now only the human ZDHHC#9 and ERF2 its homologue in yeast (Lobo *et al.*, 2002) were shown to require a co-factor for their PAT activity while fourteen human ZDHHC proteins have been shown to bear a PAT activity without a cofactor (Iwanaga *et al.*, 2009).

Yet another possibility is that the CD-MPR fails to accumulate in its acylated form despite over-expression of its appropriate PAT. Stockli and colleagues (2004) showed that the S-palmitoylation of the CD-MPR occurs in endosomes and at the plasma membrane where only approximately twenty percent of the total population of the receptor is localized at steady state (Klumperman *et al.*, 1993). If the quantity of PAT for the CD-MPR endogenously expressed in HeLa cells is already enough to palmitoylate the entire endosomal CD-MPR population, then an exogenous over-expression of this PAT might not result in any additional increase of the level of S-palmitoylation of the receptor. Finally, it is also possible that the CD-MPR is S-palmitoylated by a mechanism that does not involve the ZDHHC protein family. For example, the level of S-palmitoylation of Bet3, a subunit of the TRAPP complex (Menon *et al.*, 2006) is not altered by the down-regulation for multiple ZDHHC proteins in yeast (Roth *et al.*, 2006) and Bet3 can be produced in E.coli in its S-

palmitoylated form despite the fact that this bacterium strain lacks ZDHHC genes (Kim *et al.*, 2005).

In addition to the results discussed above, this work also presents an improved version of an existing protocol (Drisdel and Green, 2004) that allows to study S-palmitoylation in a semi-quantitative manner. Most notably, in this improved assay, the cleavage of palmitoyl-thioester bonds with neutral hydroxylamine and the labeling of the newly formed thiol groups with biotin are done simultaneously. This modification solves the problem of the oxidation of newly formed thiol groups which are created from S-palmitoylated sites during the incubation with neutral hydroxylamine, prior to the labeling with thiol reactive reagents. In addition, this improved assay does not require the incorporation of tritium-labeled palmitate into the S-palmitoylated proteins which requires long exposure times for the autoradiography. Therefore, we believe that this adapted assay will be a useful tool to study the S-palmitoylation not only of the CD-MPR but also of other proteins.

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Tables

Table 1: Genes, mRNAs and proteins of the human ZDHHC family

NCBI Gene symbol (Gene ID)	ZDHHC mRNA[a]	Protein Isoform (RefSeq accession number)
ZDHHC1 (29800)	NM_013304.2	ZDHHC#1 (NP_037436.1)
ZDHHC2 (51201)	NM_016353.4	ZDHHC#2 (NP_057437.1)
ZDHHC3 (51304)	NM_001135179.1 NM_016598 NM_001135180.1	ZDHHC#3_1 (NP_001128651.1) ZDHHC#3_2 (NP_057682.1) ZDHHC#3_3 (NP_001128652.1)
ZDHHC4 (55146)	NM_001134387.1 NM_001134388.1 NM_018106.3 NM_001134389.1	ZDHHC#4 (NP_001127861.1) same same same
ZDHHC5 (25921)	NM_015457.2	ZDHHC#5 (NP_056272.2)
ZDHHC6 (64429)	NM_022494.1	ZDHHC#6 (NP_071939.1)
ZDHHC7 (55625)	NM_001145548.1 NM_017740.2	ZDHHC#7_1 (NP_001139020.1) ZDHHC#7_2 (NP_060210.2)
ZDHHC8 (29801)	NM_013373.2	ZDHHC#8 (NP_037505.1)
ZDHHC9 (51114)	NM_016032.2 NM_001008222.1	ZDHHC#9 (NP_057116.2) same
ZDHHC11 (79844)	NM_024786.2	ZDHHC#11 (NP_079062.1)
ZDHHC12 (84885)	NM_032799.4	ZDHHC#12 (NP_116188.2)
ZDHHC13 (54503)	NM_019028.2 NM_001001483.1	ZDHHC#13_1 (NP_061901.2) ZDHHC#13_2 (NP_001001483.1)
ZDHHC14 (79683)	NM_024630.2 NM_153746.1	ZDHHC#14_1 (NP_078906.2) ZDHHC#14_2 (NP_714968.1)
ZDHHC15 (158866)	NM_144969.2 NM_001146256.1	ZDHHC#15_1 (NP_659406.1) ZDHHC#15_2 (NP_001139728.1)
ZDHHC16 (84287)	NM_032327.2 NM_198046.1 NM_198043.1 NM_198045.1	ZDHHC#16_1 (NP_115703.2) same ZDHHC#16_2 (NP_932160.1) ZDHHC#16_3 (NP_932162.1)
ZDHHC17 (23390)	NM_015336.2	ZDHHC#17 (NP_056151.2)
ZDHHC18 (84243)	NM_032283.1	ZDHHC#18 (NP_115659.1)
ZDHHC19 (131540)	NM_001039617.1	ZDHHC#19 (NP_001034706.1)
ZDHHC20 (253832)	NM_153251.2	ZDHHC#20 (NP_694983.2)
ZDHHC21 (340481)	NM_178566.3	ZDHHC#21 (NP_848661.1)
ZDHHC22 (283576)	NM_174976.2	ZDHHC#22 (NP_777636.2)
ZDHHC23 (254887)	NM_173570.3	ZDHHC#23 (NP_775841.2)
ZDHHC24 (254359)	NM_207340.1	ZDHHC#24 (NP_997223.1)

[a] RefSeq accession number at the NCBI

Table 2: Detection of the expression of the human ZDHHC protein by RT-PCR in HeLa cells

mRNA assayed	Primer pair[a]	Annealing condition	Expression detected[b]
ZDH#1	zdh1.694.up zdh1.364.dw	42℃ 40 seconds	No
ZDH#2	ZDH2-up-445 ZDH2-dw-287	50℃ 30 seconds	Yes
ZDH#3_1 **ZDH#3_3**	ZDH3a.624.up ZDH3a.378.down	55℃ 30 seconds	Yes
ZDH#3_2	None		ND
ZDH#4	ZDH4.523.up ZDH4.278.down	55℃ 30 seconds	Yes
ZDH#5	ZDH5.995.up ZDH5.794.down	55℃ 30 seconds	Yes
ZDH#6	ZDH6.624.up ZDH6.447.down	55℃ 30 seconds	Yes
ZDH#7_1	None		ND
ZDH#7_2	None		ND
ZDH#8	ZDH8.469.up ZDH8.283.down	55℃ 30 seconds	Yes
ZDH#9	ZDH9.917.up ZDH9.740.down	55℃ 30 seconds	Yes
ZDH#11	None		ND
ZDH#12	zdh12.265.up zdh12.68.dw	42℃ 40 seconds	No
ZDH#13_1 **ZDH#13_2**	ZDH13.1395.up ZDH13.1245.down	55℃ 30 seconds	Yes
ZDH#14_1	None		ND
ZDH#14_2	None		ND
ZDH#15_1 **ZDH#15_2**	zdh15.380.up zdh15.82.dw	42℃ 40 seconds	Yes
ZDH#16_1	None		ND
ZDH#16_2	None		ND
ZDH#16_3	None		ND
ZDH#17	ZDH17.303.up ZDH17.82.down	55℃ 30 seconds	Yes
ZDH#18	ZDH18-up-834 ZDH18-dw-382	50℃ 30 seconds	Yes
ZDH#19	zdh19.276.up zdh19.99.dw	42℃ 40 sec	No
ZDH#20	ZDH20-up-1033 ZDH20-dw-662	50℃ 30 seconds	Yes
ZDH#21	zdh21.562.up zdh21.328.down	42℃ 40 seconds	No
ZDH#22	None		ND
ZDH#23	None		ND
ZDH#24	ZDH725.723.up ZDH725.550.down	55℃ 30 seconds	Yes

[a] All primer sequences are provided as supplemental data in the Table 4.

[b] Detection of a specific single amplification product after RT-PCR using HeLa cDNA as a template. ND: not determined.

Table 3: Intracellular localization of the human ZDHHC proteins in HeLa cells

Proteins	Intracellular localization
ZDHHC#1	Endosomes + PM
ZDHHC#2	Endosomes + PM
ZDHHC#3_1 /#3_2 /#3_3	Golgi /ND /ND
ZDHHC#4	ER
ZDHHC#5	ND
ZDHHC#6	ND (likely ER)[a]
ZDHHC#7_1 /#7_2	Endosomes /ND
ZDHHC#8	ND
ZDHHC#9	ND
ZDHHC#11	Endosomes + PM
ZDHHC#12	ND
ZDHHC#13_1 /#13_2	Golgi /ND
ZDHHC#14_1 /#14_2	ND /ND
ZDHHC#15_1 /#15_2	Endosomes + PM /ND
ZDHHC#16_1 /#16_2 /#16_3	Golgi /ND /ND
ZDHHC#17	Golgi
ZDHHC#18	Endosomes + PM
ZDHHC#19	ND
ZDHHC#20	Endosomes + Golgi
ZDHHC#21	Golgi
ZDHHC#22	ND
ZDHHC#23	ND
ZDHHC#24	ER

ER: endoplasmic reticulum; PM: plasma membrane; Golgi: Golgi apparatus; ND: not determined. [a] ZDHHC#6 contains the ER recycling motif –KKxx and therefore most likely localizes in the ER.

Figures

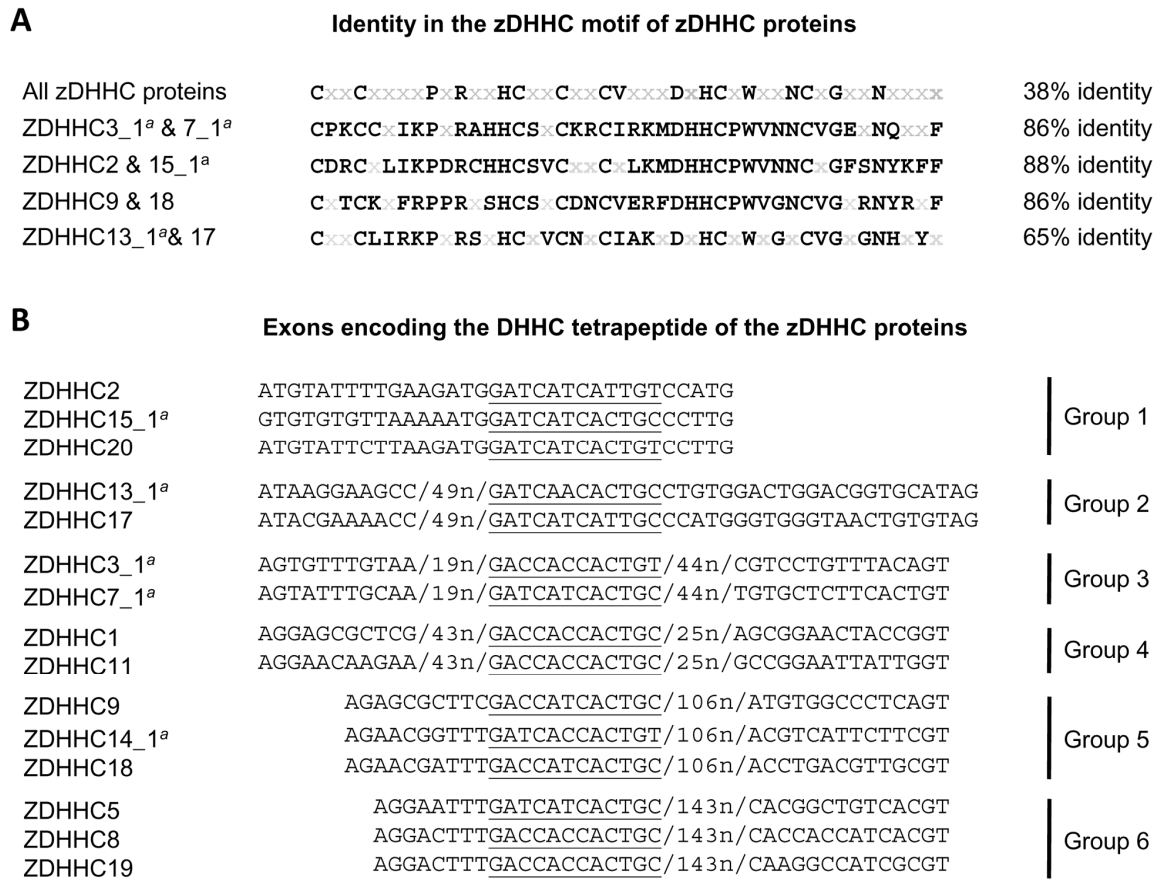

Figure 1: The ZDHHC genes form seven distinct phylogenetic groups

The primary amino acid sequences of the human ZDHHC proteins and the structure of the exons encoding the ZDHHC motifs of the human ZDHHC proteins were compared to establish a phylogenetic classification of the human ZDHHC genes.

(A) Consensus sequences resulting from the alignment of a forty-four amino acid region encompassing the ZDHHC motif of the indicated human ZDHHC proteins. (B) The ZDHHC genes are grouped on the basis of the structural similarities (size and genomic position) of the exon encoding the ZDHHC motif. The nucleotide sequence coding the DHHC tetra-peptide is underlined. [a] The isoform(s) of this proteins share the same sequence. n: nucleotide.

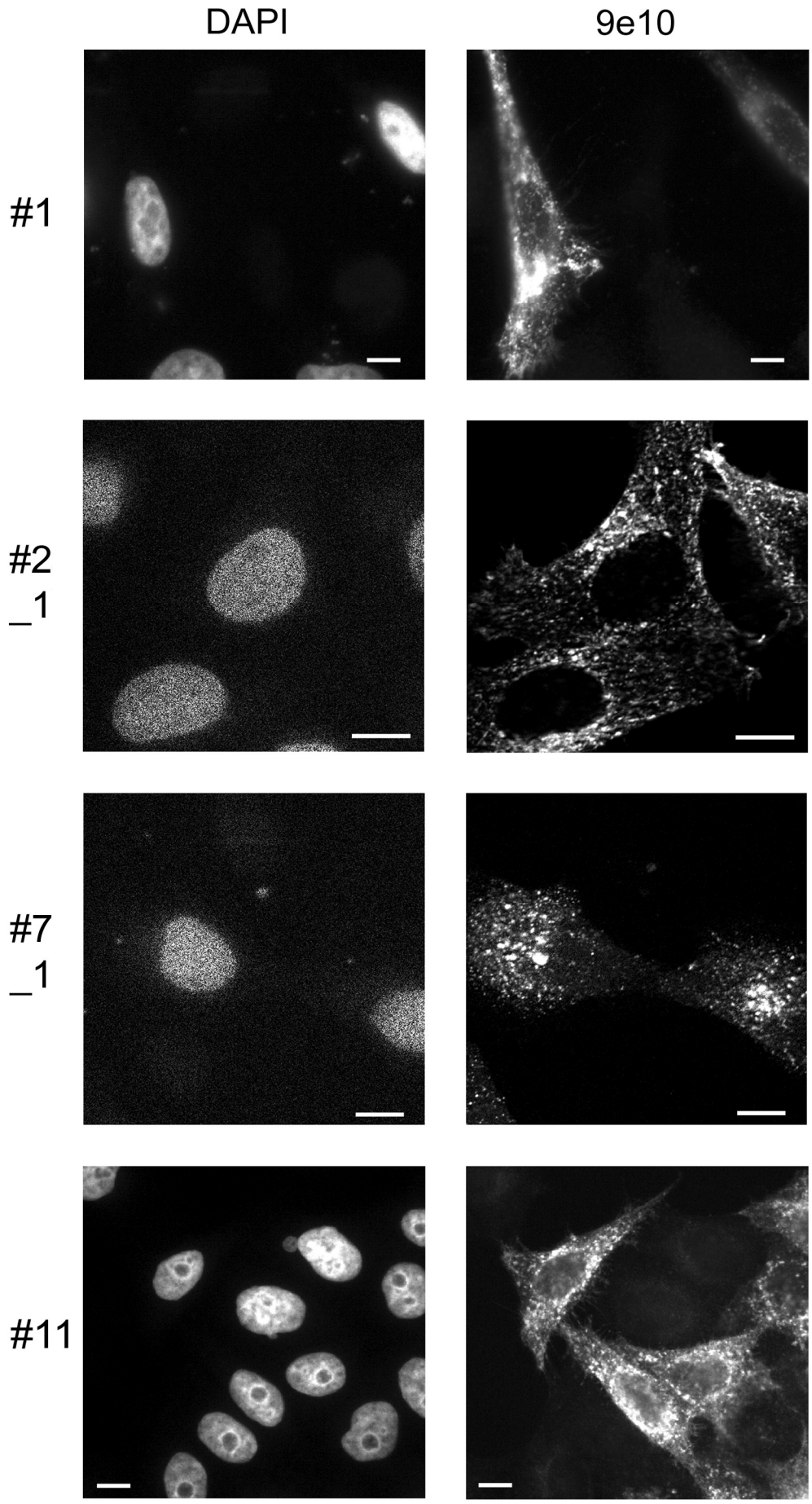

Figure 2 (1/4)

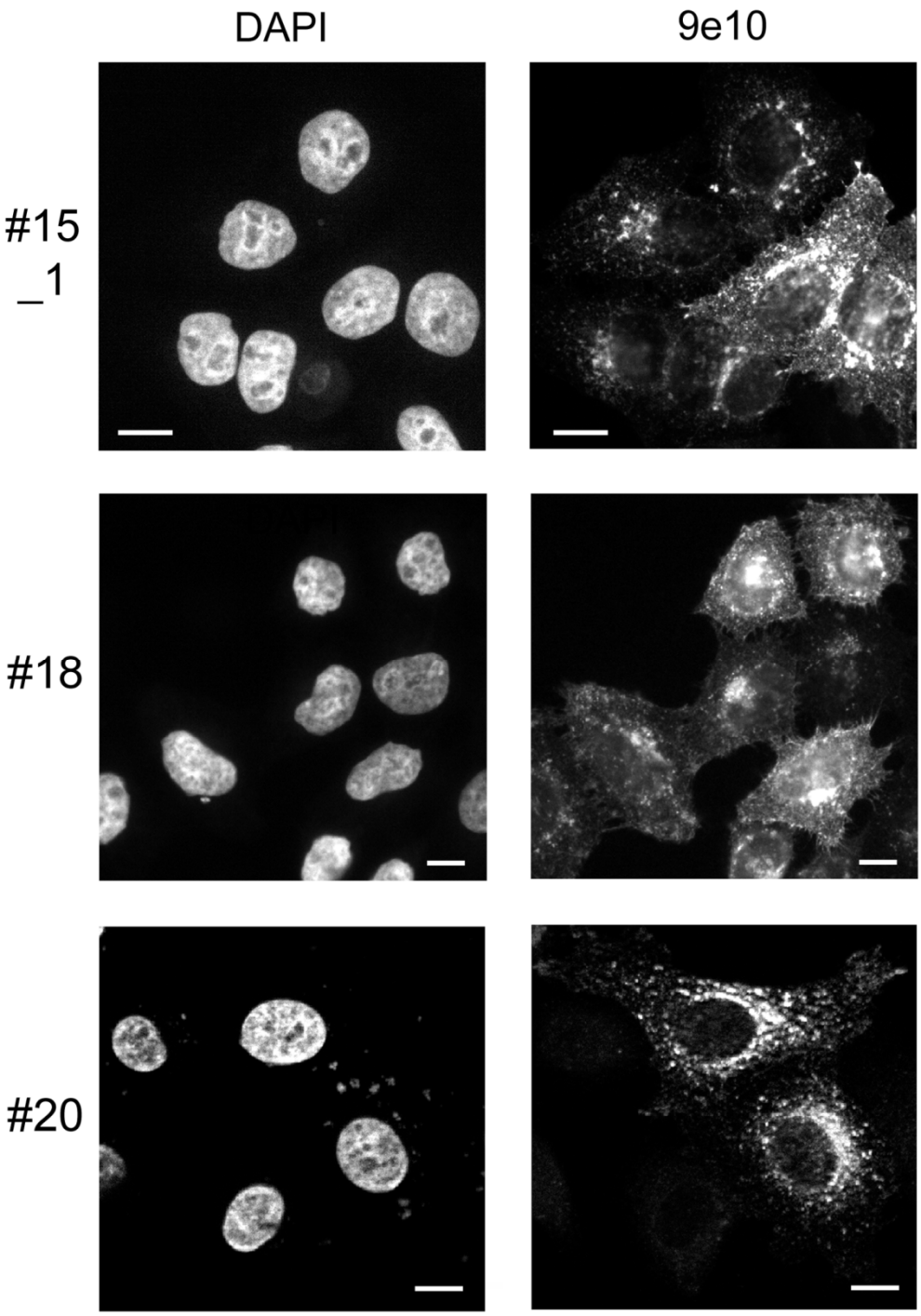

Figure 2 (2/4)

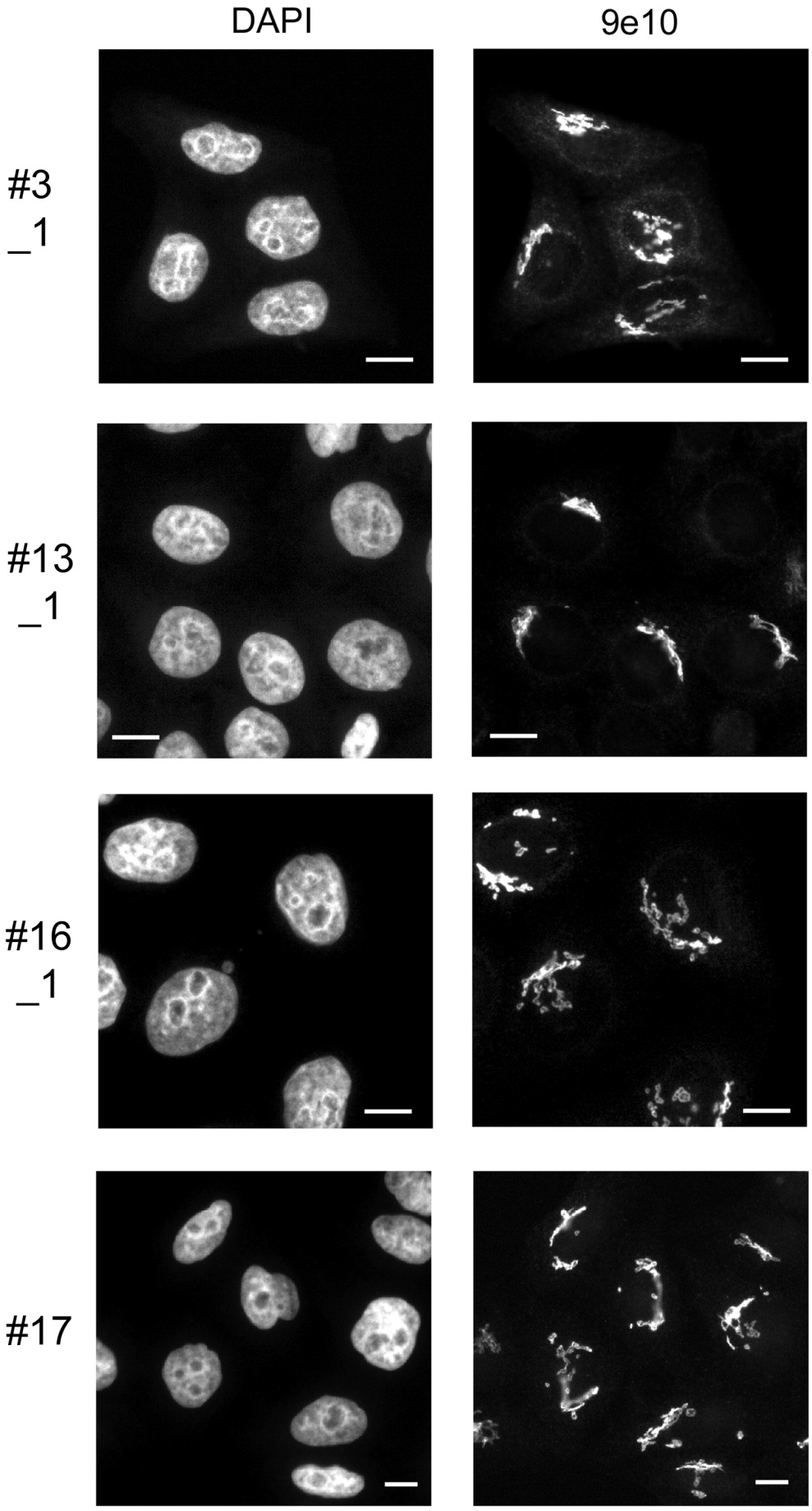

Figure 2 (3/4)

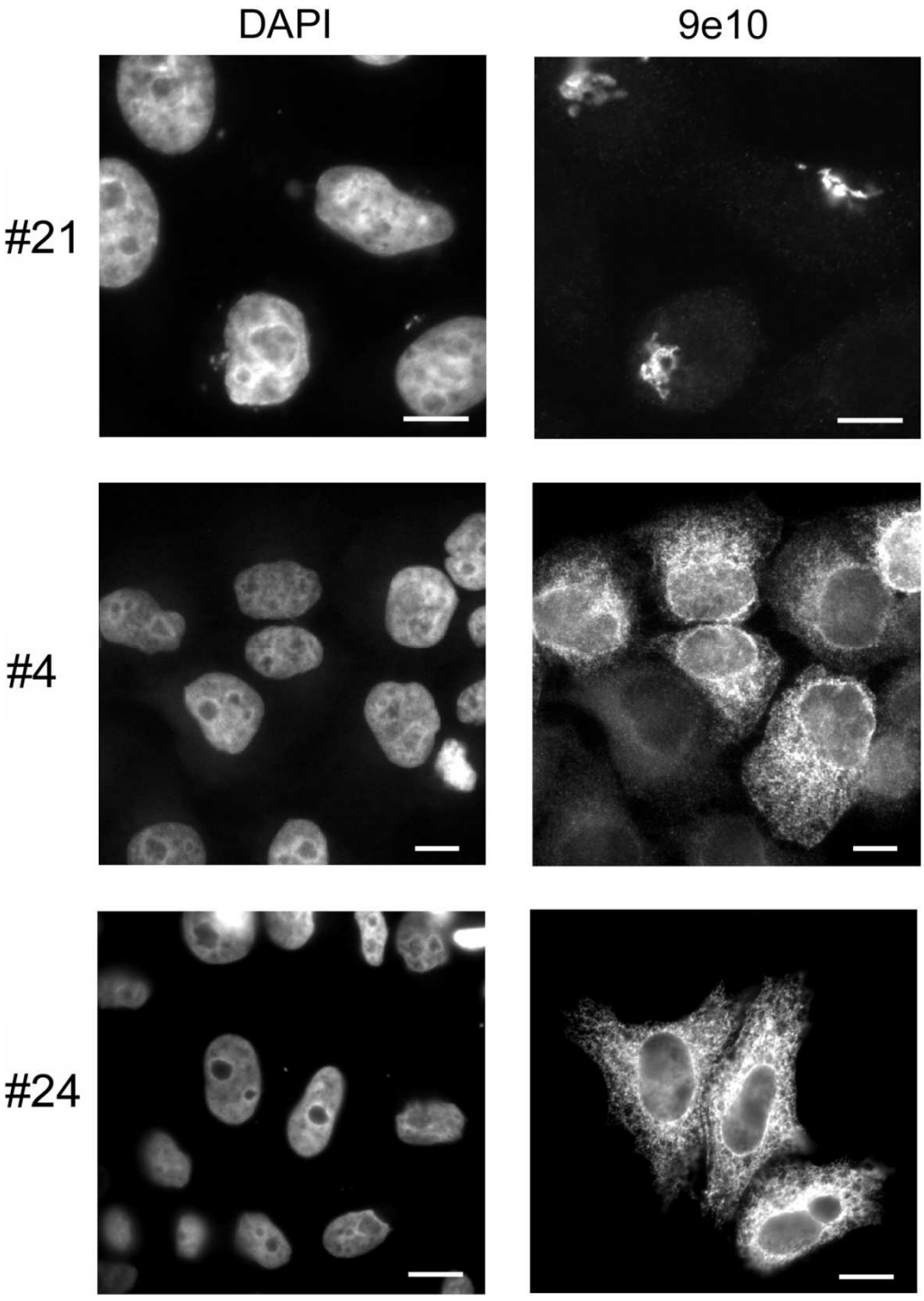

Figure 2 (4/4): Localization of ZDHHC proteins in HeLa cells by fluorescence microscopy.

A N-terminal myc tagged form of various human ZDHHC proteins (indicated here by their number) were transiently expressed in HeLa cell by transfection. After three days of culture, the transfected cells were analyzed by immuno-fluorescence using DAPI to stain DNA and the monoclonal antibody 9e10 to detect myc-tagged ZDHHC proteins. The white bar embedded within each picture represents 10 µm.

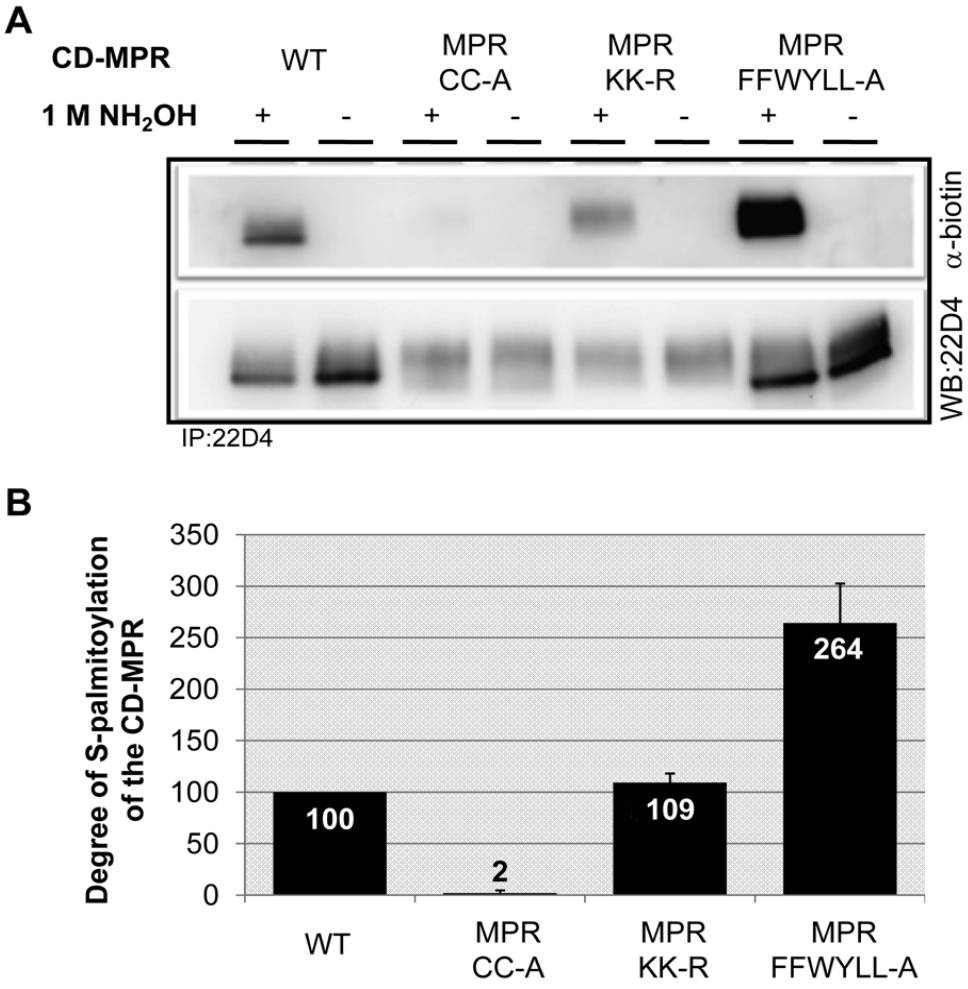

Figure 3: Determination of the level of S-palmitoylation of the CD-MPR in HeLa cells

The ABE assay was used with HeLa cells stably transfected either with a wild type form of the bovine CD-MPR (WT), with a palmitoylation deficient CD-MPR mutant (MPR CC-A), with an ubiquitination deficient CD-MPR mutant (MPR KK-R) or with an over-palmitoylated CD-MPR mutant (MPR FFWYLL-A). (A) Results of western blot with or without 1 M hydroxylamine treatment during the ABE assay. Upper blot: immuno-detection of biotin. Lower blot: after the immuno-detection of biotin, the western blot membrane was stripped and the various forms of the CD-MPR were subsequently immuno-detected to determine the respective expression level. (B) Quantification of the degree of S-palmitoylation of various forms of the CD-MPR stably transfected in HeLa cells (% of the degree of S-palmitoylation of the wild type bovine CD-MPR stably transfected in HeLa cells). The error bars represent the standard deviation values calculated from the total number of quantifications performed.

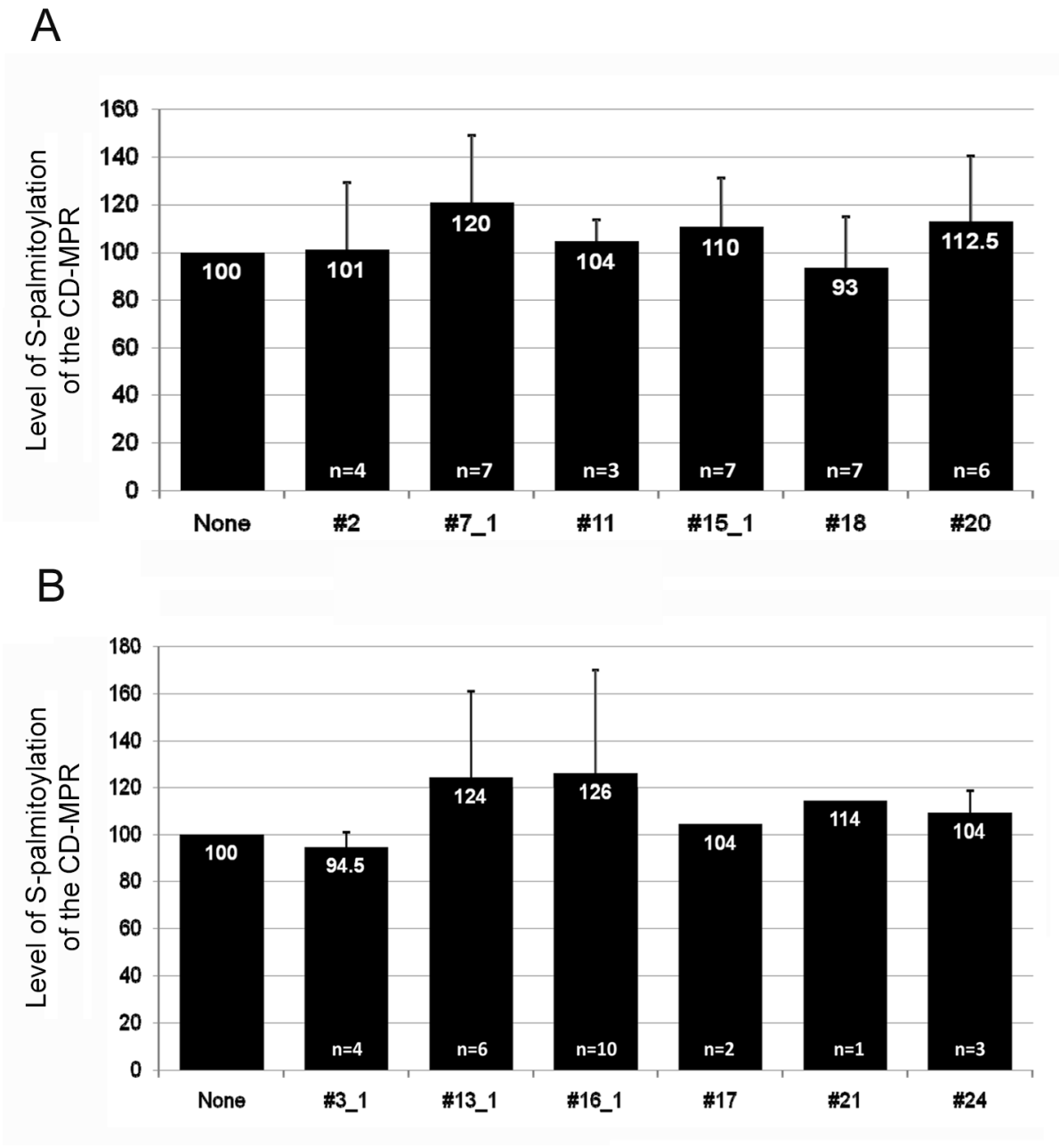

Figure 4: The effect of over-expression of individuals ZDHHC proteins on the level of S-palmitoylation of the CD-MPR in HeLa cells

The effect of the transient over-expression of different human ZDHHC proteins on the degree of S-palmitoylation of the wild type bovine CD-MPR stably transfected in HeLa cells. (A and B) Quantification of the level of S-palmitoylation of the wild type bovine CD-MPR in HeLa cells transiently transfected with the human ZDHHC proteins indicated by their number (the level of S-palmitoylation of the wild type bovine CD-MPR in untransfected HeLa cells was set as 100%). N: number of quantifications performed. The error bars represent the standard deviation values calculated from the total number of quantifications performed.

Supplemental data

Table 4: Primer list

Primer	5´- 3´ sequence
zdh1.694.up	GCAGGAACACGAACCACA
zdh1.364.dw	TTCAACCGAAGCCAGCAC
ZDH2-up-445	ATTTATCACAGACGGAGCA
ZDH2-dw-287	AGAGAGAGCCAAGAGGAGA
ZDH3a.624.up	GAAGGAGCTGCACTTTGTCC
ZDH3a.378.down	GTACAAGTGCCCCAAATGCT
ZDH4.523.up	AACGGTGCACACACCAGTTA
ZDH4.278.down	AGCTGGAGTTGTCCTTGCAT
ZDH5.995.up	GTTGGCTCCTTCAAGCTGTC
ZDH5.794.down	CAGACCTGAGCCGTTACACA
ZDH6.624.up	TAATCCCAAGGCAAACAAGG
ZDH6.447.down	GGGTTGTATCCATGCTGCTT
ZDH8.469.up	TGAGTGACAGCAGGAACAGG
ZDH8.283.down	GATGTGCGAGGTATCCAGGT
ZDH9.917.up	TCCTCCAGTGGCAAAATACC
ZDH9.740.down	CTTTCCTCGTGGCTCTCAAC
zdh12.265.up	TGGCTGTCTGCTCCTCTT
zdh12.68.dw	GAATCACGCTGGTGCTCT
ZDH13.1395.up	AAAACCTATGCACCGTCCAG
ZDH13.1245.down	CCTTGCAGAAACTGGCTCTC
zdh15.380.up	GCTCCACTTCCAGTTCTTGT
zdh15.82.dw	ATTGTCCTCGTCGTGCTCT
ZDH17.303.up	GTTATTGATGGCAGCCCAAT
ZDH17.82.down	CTCCACCCAGAGGAAATCAA
ZDH18-up-834	GCTTGCTGGTGTCTCCTT
ZDH18-dw-382	GCTGCCATCCTCTTCTTC
zdh19.276.up	AGCGGAGCCTTGATGTAA
zdh19.99.dw	CAATGTGGTGCTGCTGGT
ZDH20-up-1033	CTCCATTCTCCAGCCACT
ZDH20-dw-662	TCATCAGCGTCCTCTCACT
zdh21.562.up	TGCCCATAAAGGCTGCTAGT
zdh21.328.down	GGCCACTGTGTGAGGAGAAT
ZDH725.723.up	GGGACCCAGGTCATAGGAGT
ZDH725.550.down	TTGCTCACAGGCAGAGTGTC

General discussion

S-palmitoylation regulates the transport of the CD-MPR from early endosomes to the trans-Golgi network

In order to understand the biological role of a given protein, it is often required to precisely know the mechanisms sustaining its intracellular trafficking. In the specific case of the CD-MPR, research of the last twenty years only partially uncovered the mechanisms sustaining its intracellular trafficking. As previously mentioned, it was found that some amino acid residues of the cytosolic tail of the receptor can interact with different adaptins (e.g. AP-2 and GGA1), and it was clearly established that these interactions allow the receptor to be incorporated into transport vesicles (Dahms and Hancock, 2002). In addition, other amino acid residues of the cytosolic tail of the CD-MPR were found to be important for the trafficking of the receptor from the endosomes to the trans-Golgi network ($F^{18}W^{19}$ and C^{34}), but the precise mechanisms in which they are involved are still unknown. In particular, the cystein residue at the position thirty-four of the cytosolic tail of the CD-MPR prevents the trafficking of the receptor to lysosomes, but it is not known how (Schweizer *et al.*, 1996). Interestingly, this cystein residue is enzymatically S-palmitoylated in endosomes and at the plasma membrane (Stockli and Rohrer, 2004). It is likely that the attachment of a bulky highly hydrophobic moiety which would be inserted in the membrane induces a drastic conformational change of the cytosolic tail of the CD-MPR, which could modulate the presentation of trafficking motifs, thus influencing the intracellular trafficking of the receptor. Another hypothesis is that S-palmitoylation induces the segregation of the receptor into specialized membrane sub-domains which would be enriched in proteins required for its intracellular transport.

In this work, we demonstrated that S-palmitoylation induces the association of the CD-MPR with cholesterol rich membrane sub-domains. In addition, we give evidence that those cholesterol rich membrane sub-domains are neither at the plasma membrane nor in late endosomes. Finally, we quantified the CD-MPR population associated with cholesterol rich membrane sub-domains, and found that the amount of CD-MPR associated with cholesterol rich membrane sub-domains is similar to the amount of the CD-MPR previously found to be associated with endosomes (Klumperman *et al.*, 1993). Therefore, all those results suggest that S-palmitoylation induce the segregation of the CD-MPR into cholesterol rich membrane sub-domains localized in early endosomes. Interestingly, Grimmer and colleagues (2000) gave evidence that the transport of the CD-MPR from the endosomes to the trans-Golgi network depends on cholesterol rich membrane sub-domains. Therefore, the results presented in this work strongly suggest that S-palmitoylation sustains the retrieval of the CD-MPR from the endosomes to the trans-Golgi network by promoting the association of the receptor with cholesterol rich endosomal membrane sub-domains at the level of early endosomes. It is not known yet, whether cholesterol rich membrane sub-domains sustain directly or indirectly the trafficking of the CD-MPR at the endosomal level. As previously mentioned, there is evidence that cholesterol rich membrane sub-domains also play an important role in the transport of various viruses, toxins and proteins from the endosomes to the trans-Golgi network (Bonifacino and Rojas, 2006). Once again, are cholesterol rich membrane sub-domains directly or indirectly sustaining the transport of these biomolecules? It would be interesting to characterize endosomal cholesterol rich membrane sub-domains to find out whether they are specifically enriched or not in proteins sustaining the intracellular transport of biomolecules. Interestingly, McCormick and colleagues (2008) recently found that the subunit of

the retromer VPS26 partially associates with cholesterol rich membrane sub-domains. Are the cholesterol rich endosomal membrane sub-domains also containing adaptins and coat proteins? The answer to this question will help to determine the role of cholesterol rich endosomal membrane sub-domains in the transport of biomolecules from the endosomes to the trans-Golgi network.

As previously mentioned, the CD-MPR is enzymatically S-palmitoylated. Therefore, by modifying the CD-MPR with a palmitoyl moiety, and thus promoting the association of the receptor with endosomal cholesterol rich membrane sub-domains, the protein acyl transferase for the CD-MPR seems to directly control the transport of the receptor from the early endosomes to the trans-Golgi network. Interestingly, a similar phenomenon has already been shown for the intracellular transport of H-Ras and N-Ras. There is evidence that S-palmitoylation regulates the transport of those two small GTPases from internal membranes (e.g. Golgi) to the plasma membrane (Goodwin *et al.*, 2005; Rocks *et al.*, 2005). However, unlike for H-Ras and N-Ras, the protein acyl-transferase of the CD-MPR has not been identified yet. Therefore, the subsequent identification of this protein acyl transferase will help to further understand the regulation of the trafficking of the CD-MPR throughout the cell.

Only small amounts of CD-MPR transit through late endosomes in HeLa cells

As previously mentioned, the localization of the CD-MPR within endosomes has been studied by electron microscopy, and a small percentage of the receptor was found in endosomal structures which have the morphological appearance of late endosomes (Klumperman *et al.*, 1993). This observation led to the hypothesis that the CD-MPR transits through late endosomes during its trafficking throughout the cell.

In this work, the CD-MPR could not be detected in late endosomes purified from BHK cells. This discrepancy could be explained by the fact that, in the work of Klumperman and colleagues, endosomal structures were classified as early or late endosomes on the basis of their morphological characteristics, whereas, in this PhD thesis, late endosomes were identified by their specific enrichment with LBPA which is a lipid specifically enriched in late endosomes (Kobayashi *et al.*, 1998a). It is thus possible that some of the endosomal structures identified as late endosomes in the work of Klumperman and colleagues were actually maturing multi-vesicular bodies which already had the morphological characteristics of late endosomes but did not acquire their biochemical features yet. Although this discrepancy could also result from the fact that different cells were used in this work compared to the study of Klumperman and colleagues, our observation suggests that the CD-MPR rarely transits through late endosomes during its trafficking throughout the cell.

Additional controversial data suggest that it is at the level of the late endosomes that the CD-MPR is normally retrieved from the endosomes to the trans-Golgi network (Diaz and Pfeffer, 1998). However, the fact that the CD-MPR could not be detected in late endosomes of BHK cells suggests that the retrieval of the CD-MPR from the endosomes to the trans-Golgi network mainly occurs at the level of the early endosomes, and that the retrieval of the receptor from the late endosomes would be a rare event. This would imply that the majority of the endosomal CD-MPR population is retrieved to the trans-Golgi network from the early endosomes, whereas an additional transport mechanism also exists at the level of the late endosomes to rescue the CD-MPR which failed to be previously retrieved. Such a two-step retrieval mechanism would ensure that very little of the CD-MPR moves along the endosomal pathway to the lysosomes where it would be quickly degraded. These redundant retrieval mechanisms would be a feature of the

trafficking of the CD-MPR that could explain the very long half-life of the receptor which is more than forty hours (Rohrer *et al.*, 1995), despite the fact that the receptor rapidly cycles through the endocytic pathway to deliver newly synthesized lysosomal enzymes to the lysosomes (Duncan and Kornfeld, 1988).

Ubiquitination, a mechanism to down-regulate damaged membrane proteins from the plasma membrane and endosomes?

As already mentioned, some mutant forms of the CD-MPR have a reduced half-life (Rohrer *et al.*, 1995; Breuer and Braulke, 1998). It was hypothesized that those mutant forms of the receptor miss important trafficking motifs required for their transport from the endosomes to the trans-Golgi network, which led to their accumulation in endosomes followed by a slow and unspecific delivery to lysosomes.

In this work, we show that the CD-MPR can be ubiquitinated on its cytoplasmic tail at the plasma membrane. As previously mentioned, in the context of an integral membrane protein, ubiquitination can promote internalization at the plasma membrane, and/or the transport to lysosomes for subsequent degradation. In the specific case of the CD-MPR, it is demonstrated in this work that ubiquitination is not required for efficient internalization at the plasma membrane. Therefore, is ubiquitination promoting the transport of the CD-MPR to lysosomes? It is possible that, under specific conditions, the CD-MPR could be transported to lysosomes for degradation in an ubiquitin-dependent manner. Interestingly, in their work, Breuer and Braulke (1998) gave evidence that the mutant forms of the CD-MPR that they analyzed and that have a reduced half-life seem to be degraded in a ubiquitin-dependent manner. Therefore, maybe the cell recognizes those mutant forms of the receptor as defective, and specifically directs them to lysosomes for subsequent

degradation. If this hypothesis is true, then ubiquitination of the CD-MPR would be part of a quality control mechanism. Interestingly, we observed in this work that a defective mutant form of the CD-MPR which has an aberrant plasma membrane localization is hyper-ubiquitinated, which supports the hypothesis that ubiquitination of the CD-MPR is part of a quality control mechanism.

In order to maintain its proteins in an operational state, the cell must have mechanisms to discard damaged or misfolded proteins. It is well established that quality control of proteins occurs in the endoplasmic reticulum (Ellgaard and Helenius, 2003), but there is evidence that quality control also occurs in other compartments of the secretory/endocytosis pathway. For example, misfolded cystic fibrosis transmembrane conductance regulators at the plasma membrane are targeted to lysosomes for subsequent degradation (Sharma *et al.*, 2004). The misfolded ATPase Pma1 at the plasma membrane is down-regulated in a similar manner (Liu and Chang, 2006). Interestingly, in both cases, targeting to lysosomes has been shown to occur in an ubiquitin-dependent manner.

Therefore, the result of this PhD is an additional piece of evidence arguing for the existence of a quality control mechanism for membrane proteins beyond the endoplasmic reticulum. The discovery of the regulation of this quality control mechanism as well as the detailed machinery sustaining it would greatly enhance our understanding of the homeostasis of membrane proteins within the secretory/endocytosis pathway.

ZDHHC proteins form a putative family of phylogenetically related protein acyl transferases

S-palmitoylation is a crucial post-translational modification for the activity of multiple proteins (Resh, 2006). However, the enzymology of S-palmitoylation is still poorly

understood despite the biological importance of this modification. The recent identification of several ZDHHC proteins with protein acyl transferase activity raises new possibilities to study the enzymology of S-palmitoylation (Tsutsumi *et al.*, 2008).

In this work, it was shown that most ZDHHC genes are transcribed in HeLa cells, and that ZDHHC proteins are present in multiple organelles of the secretory/endocytosis pathway. These results are in good agreement with the idea that the ZDHHC protein family could sustain the enzymology of S-palmitoylation throughout the cell. In addition, this PhD work established of a new phylogeny of the human ZDHHC gene family, and revealed six groups of ZDHHC proteins sharing a close common ancestral gene. Previous work showed that several ZDHHC proteins have similarities in there protein substrate specificities. Interestingly, these ZDHHC proteins with similar protein substrate specificities belong to the same phylogenetic groups. Therefore, ZDHHC proteins that recently evolved from a common ancestral protein seem to have similarities in their protein substrate specificities. As we have shown in this work, such knowledge might provide useful indications to study ZDHHC proteins that have not been characterized yet.

It is not clear why multiple ZDHHC proteins are protein acyl-transferases for the same protein substrates. Is this phenomenon a side effect of the recent duplications of several ancestral ZDHHC genes, or does it have a biological significance? It is tempting to speculate that ZDHHC proteins that modify the same proteins are not performing a redundant duty, but are rather effectors of independent intracellular signaling cascades. Maybe these ZDHHC proteins interact with different protein partners that would differentially modulate their enzymatic activity for the same proteins. If this hypothesis is true, then the level of S-palmitoylation of a given protein would be the result of the complex integration of multiple signals coming

from different intracellular signaling cascades. As previously mentioned, S-palmitoylation is a reversible modification that regulates the function of proteins playing important roles in multiple aspects of cellular biology, similarly to phosphorylation. Therefore, similarly to the protein kinase families, it would not be surprising that the primitive ZDHHC proteins evolved to form a large and complex family of proteins which modify multiple protein substrates, and are themselves under the control of different regulatory proteins. The broad expression of the ZDHHC proteins, their diverse intracellular localizations and the fact that phylogenetically closely related ZDHHC proteins share similar protein substrates are all elements that support this hypothesis. Therefore, the extensive characterization of ZDHHC proteins and their regulators should be the priority of future work attempting to clarify the enzymology of S-palmitoylation.

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